The Dissertation Committee for Shannon Laine Zandy certifies that this is the approved version of the following dissertation:

Effects of opioid antagonism on operant ethanol self-administration in adolescence and characterization of extracellular GABA in the ventral tegmental area

Committee:

Rueben A. Gonzales, Supervisor

R. Adron Harris

Michela Marinelli

Hitoshi Morikawa

Richard A. Morrisett

Effects of opioid antagonism on operant ethanol self-administration in adolescence and characterization of extracellular GABA in the ventral tegmental area

by

Shannon Laine Zandy, Pharm.D.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2016

Dedication

This dissertation is dedicated to Dr. Francis Allan Wiggle and Dixie Nelle Wiggle.

Acknowledgements

I would like to acknowledge my dissertation committee members for their service, time, and support of my doctoral candidacy: Rueben A. Gonzales, Ph.D., R. Adron Harris, Ph.D., Micky Marinelli, Ph.D., Hitoshi Morikawa, Ph.D., and Richard A. Morrisett, Ph.D.

I would like to acknowledge my scientific mentors: Joanna Peris, Ph.D., for introducing me to research and sending ongoing encouragement; Doug Matthews, Ph.D., for furthering my training and always providing valuable perspective; and finally, Rueben A. Gonzales, Ph.D., my graduate supervisor, for all of his support and guidance over the years.

I would like to acknowledge my colleagues for their scientific discussions, friendship and enduring patience: Ashley Vena, Roberto Cofresi, James Reno, Ph.D., Christy Schier, Ph.D., Jennifer Carrillo, Ph.D., Esther Maier, Ph.D., Regina Mangieri, Ph.D., Armando Salinas, Ph.D., James Doherty, Ph.D., Neha Thakore, Pharm.D., John Valenta, Ph.D.

Finally, I would like to acknowledge my family and friends, especially my husband, Adley Hemphill, for always supporting me to achieve my goals.

Effects of opioid antagonism on operant ethanol self-administration in adolescence and characterization of extracellular GABA in the ventral tegmental area

Shannon Laine Zandy, Ph.D.

The University of Texas at Austin, 2016

Supervisor: Rueben A. Gonzales

The endogenous opioid peptide system is hypothesized to be involved in ethanol self-administration and relapse behaviors. Naltrexone, a nonselective opioid antagonist, is an approved medication for alcohol use disorder which has been shown to decrease drinking in adult animal models and select clinical populations, but little is known about the efficacy of naltrexone in animal models that begin drinking ethanol in adolescence. Therefore, we investigated the effects of systemic naltrexone administration in an adolescent rat model of operant ethanol self-administration. We found that naltrexone significantly reduced ethanol intake and motivation to obtain ethanol in adolescent and adult rats. Following a period of abstinence, naltrexone also significantly reduced "relapse" to alcohol seeking in both age groups. These results extend findings that naltrexone is effective at reducing ethanol intake to an adolescent animal model and

support opioid antagonism as a treatment strategy for decreasing problem drinking in late adolescents and young adults.

One potential mechanism underlying the effects of opioid receptor blockade on ethanol self-administration implicates γ-aminobutyric acid (GABA) neurons within the ventral tegmental area (VTA). Inhibitory signaling in the VTA is involved in the mechanism of action of many drugs of abuse yet there are few studies measuring extracellular GABA concentration in this region. Therefore, the remaining experiments focused on developing methods to quantify extracellular GABA in the VTA.

We first describe a novel, sensitive fluorescence method to quantify GABA concentration using high performance liquid chromatography (HPLC) of an *o*-phthalaldehyde/sulfite derivative, previously reported to produce low fluorescence not suitable for *in vivo* microdialysis applications. Next, we used quantitative microdialysis under transient conditions to characterize basal extracellular GABA concentration and the influence of uptake mechanisms in the VTA. Our results show that inhibition of GABA uptake significantly increased extracellular GABA concentration and reduced *in vivo* extraction fraction of the probe. Reduced *in vivo* extraction fraction caused significant underestimation of the increase in extracellular GABA by conventional microdialysis. Together, these results establish the foundation for future studies to investigate the regulation of extracellular GABA concentration and uptake mechanisms in the VTA in mediating the effects of ethanol, opioid antagonism and associated drug-related behaviors.

Table of Contents

List of Tables

List of Figures

List of Illustrations

Chapter 1: Background

ALCOHOL USE

Overview

Alcohol abuse and dependence are a widespread public health issue within the United States. Alcohol is the most commonly used drug with 140 million people reporting current use (SAMHSA 2015), yet we still do not fully understand the neurobiological mechanisms underlying alcohol consumption and the transition to problematic drinking or dependence. However, the prevalence of alcohol abuse is an ongoing problem, as nearly 15% of individuals meet diagnostic criteria for alcohol abuse or dependence by 18 years of age (Swendsen *et al*. 2012). In addition the negative consequences on public health, alcohol misuse cost the United States $249 billion in 2010, and 77% of those costs were due to binge drinking (Sacks *et al*. 2015).

Adolescent alcohol use

Alcohol is the most commonly abused substance in adolescence, and approximately 16% of individuals age 12 to 20 were classified as binge drinkers in the United States (SAMHSA 2015; Witt 2010). Additionally, over 6% of adolescents meet criteria for alcohol dependence (Swendsen *et al*. 2012). Adolescence is a period of profound neurobiological and behavioral development, which can result in an increased

propensity to abuse alcohol (Casey and Jones 2010; Spear 2000). Indeed, a strong predictor of progression to alcohol-related problems is age of first use. Individuals who start drinking alcohol before the age of 14 were reported four times more likely to develop alcohol dependence later in life (DeWit *et al*. 2000; Grant and Dawson 1997). Additionally, individuals who begin drinking earlier in life are more likely to progress to alcohol dependence within the first ten years of drinking (Hingson *et al*. 2006). Overall, the consequences of alcohol use in adolescence have a clear and significant impact on future problems with alcohol.

Current treatment strategies

Despite the prevalence of alcohol dependence, there are only four approved pharmacological treatment options: acamprosate, disulfiram, oral naltrexone and long-acting injectable naltrexone. Disulfiram (Antabuse) inhibits acetaldehyde dehydrogenase and causes an aversive reaction when drinking alcohol. In contrast, acamprosate (Campral) and naltrexone (Revia, Vivitrol) are thought to be effective by reducing alcohol cravings (Litten *et al*. 2005). Acamprosate has been shown effective at reducing relapse to alcohol drinking (Rosner *et al*. 2010). Originally thought be a γ-aminobutyric acid (GABA) analogue, it may actually exert its effect via calcium (Spanagel *et al*. 2014). Naltrexone (Revia, Vivitrol), an opioid receptor antagonist, has been shown to reduce alcohol intake and risk of relapse in some (O'Malley *et al*. 1992; Sinclair 2001; Volpicelli *et al*. 1992), but not all clinical studies (Krystal *et al*. 2001). There is an ongoing need for development of new treatments, however only 20% of people who

abuse alcohol were reported to receive treatment of any form, including behavioral or pharmacological interventions (Grant *et al*. 2015).

While the number of adolescents diagnosed with alcohol dependence continues to increase, only a limited number of these patients receive treatment (Miranda and Treloar 2016). Recently, initial studies have shown naltrexone may also be effective at reducing heavy drinking and alcohol craving in adolescents and young adults (Miranda *et al*. 2014; O'Malley *et al*. 2015). These results are promising for designing treatment interventions to reduce alcohol abuse in adolescence as a method to prevent progression to dependence later in life.

Adolescent animal models

Animal models can be used to examine the neural correlates of alcohol use in adolescence and how these changes may be related to the risk of developing future alcohol problems. Most rodent models of alcohol use indicate adolescent animals consume more ethanol than adults, similar to epidemiological data (Bell *et al*. 2011; Broadwater *et al*. 2011; Doremus *et al*. 2005; García-Burgos *et al*. 2009; Vetter *et al*. 2007). However, other studies have shown no differences or less intake in adolescents compared to adults (Schindler *et al*. 2014; Schramm-Sapyta *et al*. 2010; Siegmund *et al*. 2005). A caveat is these models utilize nonoperant self-administration paradigms. Adolescence can be characterized by hyperphagia and hyperdipsia (Nance 1983), which may influence ethanol consumption in a free access paradigm. Indeed, studies that also

monitored food and water in addition to ethanol intake found increases in overall consumption (García-Burgos *et al*. 2009; Vetter *et al*. 2007).

Recently, our lab has begun to characterize operant ethanol self-administration behavior in adolescent rats (Doherty and Gonzales 2015). This model uses sweetened ethanol to facilitate stable operant responding within the short time period of adolescence in rats. Operant self-administration models have high predictive validity when examining potential pharmacological treatments for drug abuse (Carter and Griffiths 2009). Therefore, using operant ethanol self-administration in adolescent rats can be useful to determine efficacy of currently available and future treatments on a range of alcohol drinking behaviors.

THE VENTRAL TEGMENTAL AREA

Overview

A common mechanism shared by drugs of abuse is actions on the mesocorticolimbic dopamine system, which originates in the ventral tegmental area (VTA). Ethanol affects multiple targets in the brain, but the VTA is thought to be critical in the development of ethanol-related behaviors (Gonzales et al. 2004; Koob and Volkow 2010). Dopamine neurons in the VTA are clearly involved in many drug-dependent behaviors, however recent evidence has also implicated GABA neurons in reward, aversion, and drug-dependent behaviors (Bocklisch et al. 2013; Creed et al. 2014; Fields et al. 2007; Nugent and Kauer 2008; Oliva and Wanat 2016).

The largest neuronal population (55-65%) in the VTA consists of dopamine neurons (Margolis *et al*. 2006b; Swanson 1982). There is also a substantial proportion of GABA (25-30%) and glutamate (5%) neurons (Margolis et al. 2012; Nair-Roberts et al. 2008). Additionally, recent evidence has shown some midbrain dopamine neurons can co-release GABA or glutamate (Stuber et al. 2010; Tritsch et al. 2014). The complexity of neuronal subpopulations and presence of co-release mechanisms suggests a more prominent role for neurotransmitters other than dopamine in the VTA, however future investigations are still needed to determine the physiological relevance.

In addition to heterogeneity in neuronal population, there is evidence supporting functional differences between the anterior VTA (aVTA) and posterior VTA (pVTA), first characterized by differences in GABAergic transmission (Ikemoto 2007; Sanchez-Catalan et al. 2014). For example, rats will self-administer $GABA_A$ receptor antagonists into the aVTA but not the pVTA (Ikemoto et al. 1997; Ikemoto 2005). While some of the reinforcing properties associated with infusions into the aVTA were later attributed to diffusion to the supramammillary nucleus and the anatomical border between subregions was not always distinct, these earlier studies were critical to begin to understand the complexity within the VTA. Additional work suggests a dividing line between aVTA and pVTA of approximately -5.5 mm from bregma in rats to observe functional differences (Olson et al. 2005; Sanchez-Catalan et al. 2014; Schifirneţ et al. 2014). Overall, these studies implicate heterogeneity in GABA transmission within the VTA that, in turn, may influence aspects of ethanol reinforcement.

Role in ethanol reinforcement

Given its neuroanatomical connectivity, it is not surprising the VTA is involved in mediating many drug-dependent behaviors, including ethanol reinforcement. Activation of VTA dopamine neurons is considered one important aspect of ethanol reinforcement. Ethanol increases the firing rate of VTA dopamine neurons *in vivo* and using *in vitro* preparations (Brodie *et al*. 1990; Brodie *et al*. 1999; Gessa *et al*. 1985; Okamoto *et al*. 2006). Microinjection of the D2/D3 agonist quinpirole into the VTA reduces operant responding for ethanol (Hodge *et al*. 1993). Rats will also directly self-administer ethanol into the pVTA, but not the aVTA, which has been suggested to depend on activation of VTA dopamine neurons (Rodd-Henricks *et al*. 2000; Rodd *et al*. 2004). Additionally, the activity of dopamine neurons in the VTA changes during development across adolescence, which may have important implications on early onset of problematic alcohol use. Dopamine neuron firing displays an inverted U-shaped curve, with the peak around puberty in rats (P45) (McCutcheon and Marinelli 2009; McCutcheon *et al*. 2012).

The role of dopamine in ethanol reinforcement has been extensively examined, however ethanol has also been shown to exert actions on other signaling mechanisms within the VTA. The effects of ethanol on GABA transmission and the endogenous opioid system in the VTA will be outlined below.

Ethanol and GABA

Ethanol has been shown to induce neuroadaptive changes by modulating GABAergic plasticity in the VTA. Following a single ethanol injection (2 g/kg; i.p.) in

mice, there is a long-lasting potentiation of GABA release onto VTA dopamine neurons (Melis *et al*. 2002; Wanat *et al*. 2009). Acute ethanol (50 mM) enhanced action potential-independent GABA release in slices of the VTA (Theile *et al*. 2008; Theile *et al*. 2009). Additionally, *in vivo* firing of VTA GABA neurons increased following acute low dose noncontingent intravenous ethanol administration and also during presentation of cues that predicted ethanol availability in a runway paradigm (Steffensen *et al*. 2009). Furthermore, chronic ethanol administration has been shown to enhance VTA GABA neuron firing rate (Gallegos *et al*. 1999) and increased spontaneous inhibitory postsynaptic currents (IPSCs) onto VTA dopamine neurons were recently observed following ethanol self-administration in adolescence (Schindler *et al*. 2016).

Conversely, in other reports acute ethanol has been shown to reduce VTA GABA neuron firing rate (Gallegos *et al*. 1999; Stobbs *et al*. 2004). Ye and colleagues reported ethanol (40 mM) application inhibited the firing rate of VTA GABA interneurons in slices and decreased GABA release (Xiao *et al*. 2007; Xiao and Ye 2008). However, more recently these authors concluded GABA release was enhanced by ethanol in the aVTA but reduced in the pVTA showing possible subregion specificity to the acute effects of ethanol (Guan *et al*. 2012). Interestingly, it has been suggested these differences may be due to the increased density of mu opioid receptor (MOR) expression in the pVTA compared to aVTA (Mansour *et al*. 1994; Mansour *et al*. 1995). Overall, further studies using freely moving animals are still needed to determine the effects of ethanol on GABA release in the VTA.

Opioid peptide system

There are three classes of opioid receptors (mu, delta, kappa), however the most consistent evidence from animal studies implicates the mu opioid receptor (MOR) in the regulation of alcohol drinking and reinforcement. MORs are located on dendrites and axon terminals of GABAergic neurons in the VTA (Garzon and Pickel 2001; Svingos *et al*. 2001). There is also dense MOR expression on terminals from rostromedial tegmental nucleus (RMTg) GABAergic inputs to the VTA (Jalabert *et al*. 2011; Jhou *et al*. 2009). Interestingly, recent work showed MOR knockout mice display increased basal dialysate GABA concentration (Chefer *et al*. 2009a), further supporting a role of tonic opioid regulation within the VTA (Spanagel *et al*. 1992).

Most developmental studies of the opioid system have focused on gestational and early postnatal periods prior to adolescence (Georges *et al*. 1998; Spain *et al*. 1985). Across adolescence, no differences were found in MOR density in the nucleus accumbens, dorsal striatum or prefrontal cortex in Long Evans rats (Ellgren *et al*. 2008). However, another study showed MOR expression reached adult levels shortly following birth, but MOR function was blunted up to age P30 (Talbot *et al*. 2005). These authors did not test ages between P30 and adulthood, therefore it is still unclear whether there are functional alterations in the opioid system during adolescence.

Ethanol and opioids

The regulation of ethanol consumption by MORs involves multiple brain regions, including the VTA. Mice lacking MORs (Hall *et al*. 2001; Roberts *et al*. 2000) or

knockdown of MOR within the VTA (Lasek *et al*. 2007) both result in reduced ethanol intake. Microinjection of nalmefene, a nonselective opioid antagonist, into the VTA significantly reduced operant ethanol responding in alcohol-preferring (P) rats (June *et al*. 2004). Acute ethanol has been shown to increase *in vivo* endogenous opioid peptide release including within the midbrain (Jarjour *et al*. 2009; Olive *et al*. 2001), and chronic ethanol consumption increased plasma beta-endorphin levels (Zalewska-Kaszubska *et al*. 2008).

It is well accepted that nonselective opioid receptor antagonists reduce alcohol consumption and preference in animal models (Cowen *et al*. 1999; Froehlich *et al*. 1990; Mitchell *et al*. 2009; Sabino *et al*. 2013). Initial work to first show naltrexone reduced ethanol self-administration was performed in rhesus monkeys (Altshuler *et al*. 1980). Alcohol seeking behavior in rats is also reduced following opioid receptor antagonism (Ciccocioppo *et al*. 2002; Burattini *et al*. 2006). This is consistent with findings that opioid receptor antagonists attenuate ethanol-stimulated dopamine release in the nucleus accumbens during ethanol self-administration (Gonzales and Weiss 1998; Middaugh *et al*. 2003). Furthermore, recent work from our lab showed opioid receptor antagonists administered directly into the VTA reduced the delayed phase of dopamine release in the nucleus accumbens following acute ethanol administration (Valenta *et al*. 2013).

Few studies have examined whether naltrexone reduces ethanol consumption to a similar extent in adolescent animal models. Systemic naltrexone reduced two bottle choice (2BC) ethanol intake in adolescent alcohol-preferring (P) rats, with lower doses reported more effective compared to adult rats (Sable *et al*. 2006). However, there are

currently no direct comparisons between adolescent and adult rats using an operant ethanol self-administration model. Overall, these animal studies highlight part of the rationale behind use of the nonselective opioid receptor antagonist, naltrexone, to reduce relapse in human alcoholics, however the exact mechanism remains unknown (O'Malley *et al*. 1992; Volpicelli *et al*. 1992).

The original proposed mechanism for reinforcement following opioid receptor activation by ethanol and other drugs of abuse was due to inhibition of VTA GABA interneurons, which tonically inhibit dopamine neurons (Johnson and North 1992). However, MOR activation does not produce disinhibition of dopamine neuron firing in all cases and inhibition of local GABA interneurons may not be required for MOR activation of dopamine neurons (Margolis *et al*. 2003; Margolis *et al*. 2012; Theile *et al*. 2011). Additionally, MORs on RMTg GABAergic inputs to the VTA are critical for the acute actions of opiates on dopamine neurons (Jalabert *et al*. 2011; Matsui and Williams 2011; Matsui *et al*. 2014). Therefore, the two neuron model of opioid receptor reward proposed by Johnson and North (1992) in the VTA can be disputed in many cases.

Overall, both GABA and opioid signaling in the VTA are involved in ethanol reinforcement, however the direct or indirect actions of ethanol *in vivo* on these systems are still unclear. Extracellular neurotransmitter concentrations can provide valuable information on the result of changes in signaling, however the regulation of extracellular GABA concentration in the VTA has largely remain unexplored.

MICRODIALYSIS

General principles

Microdialysis is a sampling technique used to monitor neurotransmitters and other neuromodulators within the extracellular space. The microdialysis probe consists of a semipermeable membrane, often referred to as the "active area," where small molecule analytes can diffuse across into or out of the perfusate according to concentration gradient, shown in Illustration 1.1. The primary advantage of using microdialysis in neuroscience is the ability to monitor dynamic changes in basal extracellular concentrations of multiple neurotransmitters in behaving animals. In addition, the probe membrane protects the tissue from the perfusate, prevents macromolecules from crossing into the sample and can be used to simultaneously deliver drugs to the extracellular space. However, disadvantages of microdialysis include limited temporal resolution and tissue damage resulting from probe implantation. Overall, microdialysis is widely used to quantify neurotransmitters implicated in neuropsychiatric disorders such as depression, pain, and drug addiction.

In conventional microdialysis, the probe is continuously perfused with a solution (e.g. artificial cerebrospinal fluid, ACSF) and neurotransmitters in the extracellular space diffuse across the membrane into the perfusate. The rate of analyte removal from the probe exceeds the rate of analyte replacement to the membrane surface within the extracellular space at flow rates used in most experiments (Chefer *et al*. 2009b). Therefore, the concentration in the dialysate sample represents a fraction of the actual

concentration in the extracellular fluid. The ratio between the dialysate concentration and the actual extracellular concentration is termed the extraction fraction, relative recovery or probe efficiency. Despite only measuring a fraction of the extracellular concentration, conventional microdialysis is often suitable for examining changes from an averaged baseline following pharmacological manipulations. However, even if knowing the absolute extracellular concentration of an analyte is not necessary, it can be useful to draw a relationship between dialysate and extracellular concentration to use as reference point or when determining the mechanism of action of drugs.

Flow rate, membrane surface area, analyte characteristics (e.g. molecular weight), and the resistance of the tissue all influence probe recovery. Early studies used *in vitro* probe calibration to extrapolate extracellular concentration *in vivo* but this method is not always accurate because the diffusion resistance of tissue differs from the diffusion resistance in solution (Benveniste *et al*. 1989; Bungay *et al*. 1990; Chefer *et al*. 2009b). For neurotransmitters, tissue resistance is influenced by active processes such as uptake (Bungay *et al*. 1990). Changes in the physiological characteristics of the tissue such as rate of analyte clearance (e.g. uptake or metabolism) and tortuosity, as well as pharmacological treatments can lead to changes in extraction fraction during an experiment. Indeed, Parsons et al. showed that extraction fraction can be altered independent of extracellular concentration (Parsons *et al*. 1991). Therefore, dialysate measurements may not reflect changes in extracellular concentration due to the influence of extraction fraction.

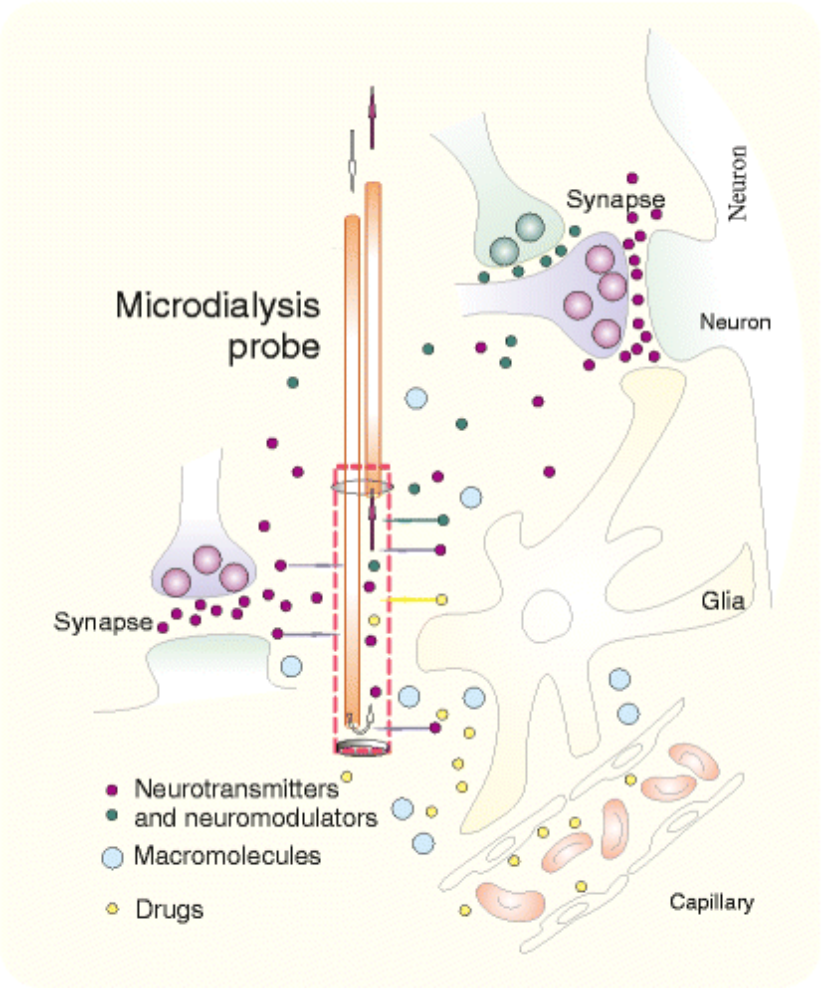

Illustration 1.1 Overview of brain microdialysis

Semipermeable membrane of probe allows neurotransmitters and neuromodulators to diffuse into perfusate. Adapted from (Anderzhanova and Wotjak 2013) with permission.

Quantitative microdialysis

Quantitative (no net flux) microdialysis was developed as a method to determine *in vivo* extraction fraction. This method includes the analyte in various concentrations in the perfusate to bracket the anticipated *in vivo* extracellular concentration (Justice 1993; Lonnroth *et al*. 1987). When the analyte concentration in the perfusate is less than the extracellular concentration, there is a net gain of analyte (via diffusion) into the dialysate sample. Similarly, when the analyte concentration in the perfusate is greater than the extracellular concentration there is a net loss of analyte from the probe. The known concentrations of analyte in the perfusate are plotted against the net gain or loss of analyte in the dialysate. The point of no net flux across the probe membrane is the *x*-intercept of the regression, and represents the extracellular concentration in the tissue (Illustration 1.2). The slope of the regression represents the *in vivo* extraction fraction. Quantitative microdialysis was also adapted for transient conditions to measure time-dependent changes of *in vivo* extraction fraction (Olson and Justice 1993). This extension of the method utilizes separate groups of animals for each analyte concentration and has been used to show *in vivo* extraction fraction can change over time following pharmacological manipulation (Cosford *et al*. 1994; Olson and Justice 1993).

The *in vivo* extraction fraction has been described as sensitive to changes in neurotransmitter clearance for monoamines (Bungay *et al*. 1990; Bungay *et al*. 2003; Morrison *et al*. 1991). For example, using pharmacological manipulations in the nucleus accumbens, it was shown that only treatments that reduced dopamine uptake but not synthesis, release or metabolism altered *in vivo* extraction fraction (Smith and Justice

1994). Additionally, 6-hydroxydopamine lesions in the nucleus accumbens reduced *in vivo* extraction fraction by approximately 50% (Parsons *et al*. 1991). Subsequent studies extended the relationship between clearance and *in vivo* extraction fraction to norepinephrine, serotonin and acetylcholine (Cosford *et al*. 1994; Vinson and Justice 1997).

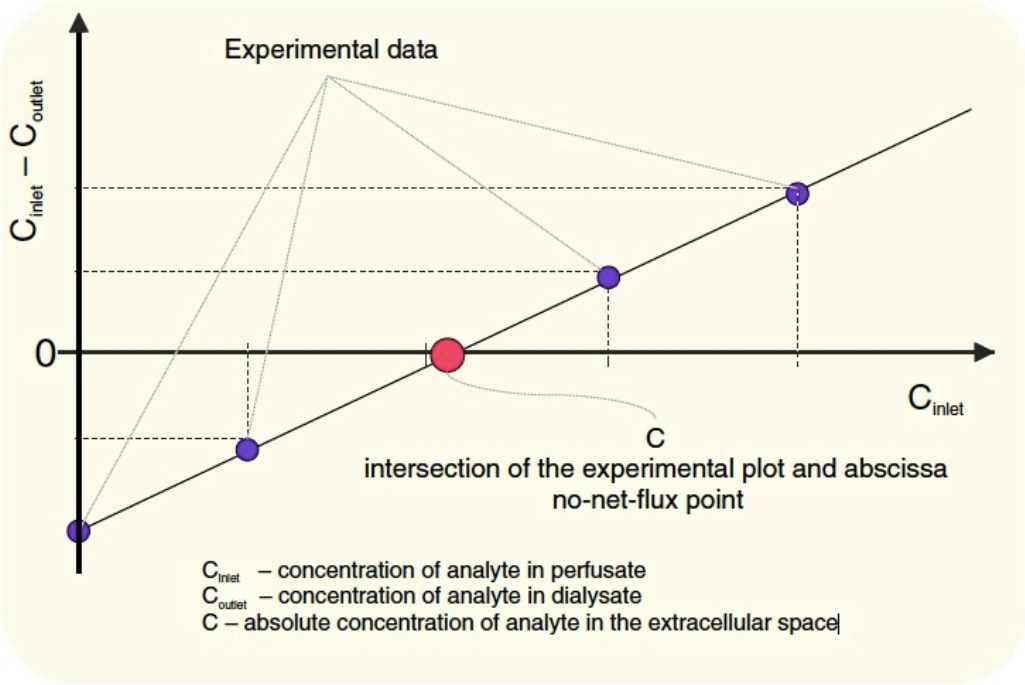

Illustration 1.2 Quantitative (no net flux) microdialysis

The *x*-intercept from the regression (red symbol) is the point of no net flux across the probe membrane and represents the extracellular concentration. Adapted from (Anderzhanova and Wotjak 2013) with permission.

The regulation of amino acid neurotransmitters glutamate and GABA in the extracellular space is largely dependent on uptake from both neurons and astrocytes (Zhou and Danbolt 2013). Therefore, whether *in vivo* extraction fraction is sensitive to changes in clearance for these neurotransmitters is still a matter of debate. Local application of the glutamate uptake blocker *trans*-pyrrolidine-2,4-dicarboxylic acid (tPDC) through the microdialysis probe significantly reduced *in vivo* extraction fraction when measured using steady-state quantitative microdialysis in the hippocampus (Chefer *et al*. 2011). However, the majority of studies suggesting *in vivo* extraction fraction represents glutamate clearance have investigated basal dynamics following chronic drug or ethanol exposure. For example, chronic ethanol administration was shown to reduce *in vivo* extraction fraction and [^{3}H]-glutamate uptake in slices (Melendez *et al*. 2005). Experiments have reported reduced *in vivo* extraction fraction either coincides with reduced (Das *et al*. 2015) or no change (Melendez *et al*. 2005; Pati *et al*. 2016) in protein expression of glutamate transporters. Indeed, a recent study reported reduced [^{3}H]-glutamate uptake in slices and *in vivo* using biosensors in the nucleus accumbens core following heroin self-administration (Shen *et al*. 2014). However, this study also reported *in vivo* extraction fraction measured using microdialysis was significantly increased. Together, these results largely suggest the relationship between *in vivo* extraction fraction and glutamate clearance remains unclear.

While there is some evidence on the regulation of glutamate in the extracellular space, there are few investigations on the regulation of extracellular GABA concentration. Quantitative microdialysis has been used to measure GABA in the nucleus

accumbens (Xi *et al*. 2003), however there are no reports of basal extracellular GABA concentration within the VTA. Additionally, inhibiting GABA transporters increases dialysate GABA concentration (Chefer *et al*. 2009a; Vihavainen *et al*. 2008) but whether changes in GABA uptake are reflected by *in vivo* extraction fraction is currently unknown.

Measuring extracellular GABA

The basal concentrations of most neurotransmitters measured using microdialysis are sensitive to the sodium channel blocker tetrodotoxin (TTX) and calcium omission from the perfusate, which meets the requirements of exocytotic release from neurons. In contrast, basal concentrations of amino acid neurotransmitters including GABA are not reliably responsive to these manipulations (Timmerman and Westerink 1997). Additionally, high affinity GABA transporters prevent most GABA from escaping the synaptic cleft into the extracellular space (Schousboe *et al*. 2013). This led to the assumption that GABA measured in microdialysis was derived from mostly non-neuronal sources. However, recent work identified specific chromatographic conditions were required for accurate detection of GABA in dialysates, emphasizing that earlier findings may have misidentified or quantified GABA with other contaminants (Rea *et al*. 2005). Under optimal conditions, extracellular GABA was responsive to TTX and calcium omission, suggesting a significant portion was of synaptic origin. These results and others have suggested that microdialysis can detect GABA from neuronal sources, although

synaptic spillover into the extracellular space is likely buffered by astrocyte networks (Del Arco *et al*. 2003; van der Zeyden *et al*. 2008).

Extracellular GABA in the VTA

Dialysate GABA concentrations in the VTA are typically reported to be lower than most brain regions, however a wide range is described in the literature (Fliegel *et al*. 2013). Despite low concentrations and the challenges in separation of GABA mentioned previously, there are studies showing acute or chronic drugs of abuse can alter dialysate GABA concentration in the VTA.

Acute 3,4,-methylenedioxymethamphetamine (MDMA) has been shown to increase dialysate GABA concentration, which was completely blocked by co-administration of TTX (Bankson and Yamamoto 2004). Nicotine administration increased VTA GABA in naïve rats but not in rats with a history of nicotine self-administation (Buczynski *et al*. 2016). Local morphine delivered through the microdialysis probe significantly reduces VTA GABA concentration (Klitenick *et al*. 1992; Sotomayor *et al*. 2005). Interestingly, the decrease in dialysate GABA concentration from systemic morphine was only observed using nipecotic acid to induce neuronal spillover (Ojanen *et al*. 2007; Vihavainen *et al*. 2008). Overall, these studies illustrate changes in dialysate GABA concentration in the VTA are implicated in the mechanism of some drugs of abuse.

Differences in GABA release following acute ethanol have been reported using electrophysiology, however microdialysis experiments have been unsuccessful in

determining *in vivo* effects of ethanol in the VTA. For example, acute ethanol injections (1 or 2 g/kg) did not alter dialysate GABA concentration in naïve or rats treated with chronic morphine injections (Kemppainen *et al*. 2010; Ojanen *et al*. 2007; Yan *et al*. 2005). Oral ethanol gavage also did not modify dialysate GABA concentration (Cowen *et al*. 1998). As mentioned above for systemic morphine, inhibiting GABA uptake is a potential method to allow better characterization within the extracellular space of whether changes in neuronal release occur following acute ethanol administration.

Overall, studies have shown changes in extracellular GABA occur following acute or chronic treatment with some, but not all, drugs of abuse. Changes in extracellular GABA concentration in the VTA may contribute to the development of drug-related behaviors. However, there is limited evidence investigating the regulation basal extracellular GABA concentration in the VTA and how these mechanisms may be involved in mediating the effects of drugs of abuse, including ethanol.

AIMS

Aim 1: To investigate the effects of systemic administration of naltrexone on operant ethanol self-administration behaviors in adolescent and adult rats

Naltrexone reduces ethanol self-administration in adult rats and some clinical populations. Significant neurobiological changes occur during development, which may influence ethanol self-administration and efficacy of treatments during adolescence. Additionally, there are few investigations using an operant ethanol self-administration model in adolescent animals. Therefore, in Aim 1 we sought to determine the effects of systemic naltrexone on operant ethanol self-administration, motivation to consume ethanol and "relapse" to ethanol seeking in rats that began drinking in adolescence compared to adulthood.

Aim 2: To develop a high sensitivity method for quantifying GABA in microdialysis samples using fluorescence detection of an OPA/sulfite derivative

A proposed mechanism for naltrexone's ability to reduce ethanol intake involves antagonism of mu opioid receptors on GABA interneurons in the VTA. Changes in inhibitory signaling have been implicated in the mechanism of drugs of abuse, including ethanol. However, there are few investigations measuring whether these changes translate to acute or chronic alterations in extracellular GABA concentration in the VTA.

Additionally, there are challenges with accurate detection of GABA in the VTA and separation using high performance liquid chromatography (HPLC). Therefore, we began developing methods to reliably quantify GABA in microdialysis samples. During this process, it became clear that fluorescence detection required less maintenance than electrochemical detection due to the high working potential necessary for electrochemical detection of GABA.

Our initial experiments conducted using electrochemical detection utilized a precolumn derivatization protocol with *o*-phthalaldehyde and sulfite to produce an electroactive GABA derivative. This protocol is often preferred to using an organic thiol as the nucleophile due to increased stability of the derivative and lack of pungent odor. However, OPA/sulfite GABA derivatives were reported to be less fluorescent than thiol derivatives and incompatible for use with sensitive applications such as *in vivo* microdialysis. Therefore, the experiments in Aim 2 were conducted to develop a reliable method to use fluorescence detection of an OPA/sulfite GABA derivative.

Aim 3: To determine if changes in GABA uptake are reflected by *in vivo* extraction fraction using quantitative microdialysis in the VTA

There is a wide range of basal GABA concentrations reported for the VTA using conventional microdialysis. Additionally, while changes in dialysate GABA concentration in the VTA have been observed following acute or chronic treatment with some drugs of abuse, it is unknown how the regulation of extracellular GABA contributes

to the interpretation of conventional microdialysis data. These factors may influence further investigation in determining the actions of ethanol on GABA concentration in the VTA using microdialysis. *In vivo* extraction fraction is considered an indirect measure of clearance for monoamine neurotransmitters, however it remains to be determined whether this is similar for amino acid neurotransmitters such as GABA. Therefore, in Aim 3 we conducted experiments to investigate the effects of inhibiting GABA uptake in the VTA on *in vivo* extraction fraction using quantitative microdialysis under transient conditions.

Chapter 2: Naltrexone reduces operant sweetened ethanol self-administration, motivation to obtain ethanol, and relapse behavior in Long Evans rats that begin drinking in adolescence or adulthood

ABSTRACT

Background: Alcohol is widely abused in adolescence and contributes to significant adverse consequences in this population. However, treatment rates for adolescents remain low in part due to lack of data on the efficacy of current medications. Naltrexone, a nonselective opioid antagonist, is effective at reducing ethanol (EtOH) consumption in adult animal models and select clinical populations. However, limited research exists on the efficacy of naltrexone in individuals who began drinking in adolescence.

Methods: Adolescent (postnatal day 36 at first EtOH exposure) and adult Long Evans rats were tested for the ability of naltrexone (0, 0.25 or 0.5 mg/kg, s.c.) to reduce intake and motivation to consume sweetened EtOH (10% sucrose + 10% EtOH; 10S10E) using a progressive ratio (PR) schedule of reinforcement. Following PR testing and 13 days of forced abstinence, rats were tested for the ability of naltrexone to reduce "relapse" EtOH seeking. Control rats drank 2% sucrose (2S) throughout the experiment.

Results: Naltrexone significantly reduced sweetened EtOH intake and motivation to obtain sweetened EtOH in both adolescent and adult rats during the PR session.

Naltrexone significantly reduced “relapse” EtOH seeking after abstinence regardless of whether self-administration began in adolescence or adulthood. Rats that began operant sweetened EtOH self-administration in adolescence pressed the lever significantly fewer times across all naltrexone doses compared to adults during the “relapse” test. Naltrexone did not alter operant behavior for sucrose in control rats.

Conclusions: Our results show naltrexone is effective at reducing sweetened EtOH intake, motivation to obtain EtOH and “relapse” behavior in an operant self-administration model using adolescent rats. Age of initiation of operant EtOH self-administration may contribute to the expression of EtOH seeking behavior. Collectively, these data support the use of naltrexone as a potential treatment option for late adolescents and young adults to reduce alcohol consumption.

INTRODUCTION

Alcohol use in adolescence is an increasing health concern as earlier age of onset is significantly associated with future rates of alcohol dependence and development of alcohol dependence at a younger age (DeWit *et al*. 2000; Hingson *et al*. 2006). The number of adolescent patients diagnosed with alcohol use disorder (AUD) is growing, however only approximately 10% receive treatment in part due to lack of data on the efficacy of currently available treatments in this population (SAMHSA 2015; Swendsen *et al*. 2012). Although research has focused on expanding pharmacotherapy treatment options for adults, little is known about whether currently available treatments are effective in adolescents (reviewed in Miranda and Treloar 2016).

Naltrexone is a nonselective opioid antagonist approved by the U.S. Food and Drug Administration for treatment of AUD in adults. In patients with alcohol dependence, naltrexone decreases relapse to heavy drinking and alcohol intake in some (O'Malley *et al*. 1992; Sinclair 2001), but not all studies (Krystal *et al*. 2001). More recently, an initial study in adolescents showed promising effects of naltrexone to reduce heavy drinking and alcohol craving (Miranda *et al*. 2014). A larger randomized clinical trial found naltrexone reduced the number of drinks during drinking episodes in young adults aged 18-25 years old (O'Malley *et al*. 2015). One challenge of clinical trials is reliance on alcohol use self-assessments, which can be more problematic if requiring adult subjects to estimate previous history of alcohol intake in adolescence. However, investigating naltrexone using relevant adolescent animal models would provide more insight into possible age-specific effects on ethanol-associated behaviors and an accurate

measure of ethanol consumption during adolescence. Operant models of alcohol use are considered the "gold standard" when testing the efficacy of a treatment because of their high predictive validity (Carter and Griffiths 2009). Operant ethanol self-administration models are common using adult animals, but rarely reported in adolescents due to the limited period of adolescence in rats (approximately 20 days in males) and initial aversive properties of ethanol. Naltrexone has been shown to reduce alcohol seeking, consumption and cue-induced reinstatement in adult rats (Burattini *et al*. 2006; Ciccocioppo *et al*. 2003; Gonzales and Weiss 1998; Hay *et al*. 2013; Henderson-Redmond and Czachowski 2014; Katner *et al*. 1999). However, whether naltrexone reduces ethanol consumption in adolescent rats using an operant self-administration model is currently unknown.

We recently developed an operant sweetened ethanol (EtOH) self-administration model in adolescent Long Evans rats (Doherty and Gonzales 2015). Here, we investigated whether systemic naltrexone administration would have similar efficacy to reduce ethanol consumption, motivation to obtain ethanol and "relapse" behavior in rats that began operant sweetened ethanol self-administration in adolescence compared to adulthood.

MATERIALS AND METHODS

Animals

Male Long Evans rats (Charles River, Raleigh, NC) arrived at postnatal day (P) 22 (43-67 g; shipped with lactating dam) or P60-65 (290-301 g). Rats were pair housed by age (25°C; 12 h light/dark schedule; lights on 0700 h) and handled daily for at least one week before training. Food and water were available ad libitum in home cages, except for brief water deprivation prior to lever press training. Following training, operant sessions occurred once per day at the same time (0900-1600 h), five days per week. Adolescent rats were at P36 at first ethanol exposure, which corresponds to early adolescence before the onset of puberty in male rats (Lewis *et al*. 2002; McCutcheon and Marinelli 2009; Spear 2000). All procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals (8th Ed., 2011) and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Drugs

Drinking solutions (2% or 10% sucrose (w/v) (2S; 10S) or 10% sucrose + 10% ethanol (v/v) (10S10E)) were made fresh twice weekly using 95% ethanol (AAPER, Shelbyville, KY), ultra-pure sucrose (MP Biomedicals, Solon, OH), and deionized water. Naltrexone hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline

for subcutaneous injection (0.25 or 0.5 mg/kg, at 1.0 ml/kg). Control saline injections were matched for volume (1.0 ml/kg).

Experimental design

Rats were separated into two drinking groups within each age group (10S10E and 2S) after the initial training phase. Following operant ethanol or sucrose self-administration (Fig. 2.1), naltrexone was injected subcutaneously 30 minutes prior to start of PR and "relapse" operant testing (defined below). The doses of naltrexone (0.25 and 0.5 mg/kg, s.c.) were chosen because previous reports showed these doses reduced operant ethanol self-administration (Czachowski and DeLory 2009; Gonzales and Weiss 1998; Hay *et al*. 2013). Naltrexone doses were assigned in a pseudo-random design between PR and "relapse" tests. We used 2S as the control reinforcing solution to approximate the amount of operant responding for 10S10E during the PR session.

Our previous data indicated a subset of adolescent rats show little or no detectable blood ethanol concentration (BEC; < 5 mg/dl) following operant ethanol self-administration at intake levels which produce detectable BECs in adult rats (Doherty and Gonzales 2015). Nondetectable BECs following operant self-administration suggested some rats may not be experiencing the central reinforcing effects of ethanol consumption and if so, could interfere with our interpretation of naltrexone data in the experiment. Therefore, we used BEC data to assign subjects into three groups (adult, adolescent, and nondetectable (ND) adolescent). The two adolescent groups were assigned using a criterion for BEC to be considered detectable (> 5 mg/dl). Data were first analyzed for

differences between adolescent BEC subgroups. If no significant differences occurred between adolescent subgroups, data were combined and analyzed based on age of onset (adolescent vs. adult) of ethanol self-administration.

Operant behavioral testing

Operant behavioral testing was conducted as previously described (Doherty and Gonzales 2015). Briefly, there were four phases in the experiment (Fig. 1). The four phases of the experiment were: 1) lever training using 10S; 2) appetitive/consummatory model; 3) fixed ratio (FR) and progressive ratio (PR) testing; and 4) "relapse" of seeking behavior after 13 days of forced abstinence in the home cage. In this study we did not extinguish operant behavior. We define "relapse" as the return of operant responding in the ethanol self-administration context after an abstinence period. The appetitive/consummatory operant model consists of a 5-minute wait period (appetitive-seeking phase), then a response requirement of 4 lever presses, followed by 20-minutes of continuous access to the drinking solution (consummatory phase). The FR2 schedule of reinforcement was implemented prior to testing on a PR schedule of reinforcement to adjust the rats to a higher response requirement and limited access to the drinking solution (10 sec for each reinforcement). The PR session was 60 minutes and breakpoint values were determined as an index of motivation to obtain the drinking solution. The progression of response requirements was similar to Walker and Koob (2007). After 13 days of forced abstinence, a 20-minute "relapse" test was performed to measure lever press responding under extinction conditions; rats could see and smell the drinking

solution, but they could not drink, and lever presses only resulted in illumination of the cue light.

Blood ethanol concentration (BEC) after operant self-administration

The protocol to measure BEC was previously described (Doherty and Gonzales 2015). Briefly, during the last three days of the appetitive/consummatory operant phase (Fig. 2.1), we sampled blood from most rats in the 10S10E group either at 12 min (allowed 5 min to drink; n=15), 22 min (allowed 15 min to drink; n=15), or 35 min (allowed 20 min to drink; n=26) after the start of the operant session. Blood was collected from the saphenous vein under light isoflurane anesthesia. The ethanol content (mg/dl) in 10 µl of blood (mixed with 90 µl saturated saline, tested in duplicate or triplicate) was measured using gas chromatography with flame ionization detection. The majority of samples were analyzed using a Scion 436 gas chromatograph (Bruker, Netherlands), Varian 8200 headspace autosampler and hydrogen generator (Model 20H-MD, Parker Hannifin, England) to produce hydrogen as the carrier gas. Data were recorded using CompassCDS (Bruker, Netherlands) software. A subset of animals (adolescents, n=9; adults, n=8) were analyzed using a previous system detailed in Carrillo et al. (2008).

Statistics

Ninety rats were included in this study. Sweetened ethanol self-administration prior to PR testing from 35 rats was previously reported in Doherty and Gonzales (2015). Data from these 35 rats were combined with new data to generate Figure 2.2. Also, BEC

data from 19 of the previously reported 35 rats were used to help classify the adolescent groups into detectable and nondetectable BEC groups as described above in the Experimental design section. Two adolescents did not establish stable self-administration and were excluded (consumption of <0.3 g/kg ethanol in 4 out of last 5 appetitive/consummatory sessions.) BECs were not sampled from 4 adults included in the behavioral analyses. Two adult rats were excluded from the BEC analysis due to lower consumption (<0.3 g/kg ethanol) only on the BEC sampling day, but they were included in later behavioral analyses. For the PR session, 2 adults in the sweetened ethanol group were excluded due to incorrect injections. For the "relapse" test, one adolescent in the sucrose control group was excluded due to unrelated death during the abstinence period.

BEC analyses

Ethanol consumption on BEC test day and BEC data were analyzed using one-way analysis of variance (ANOVA). Pearson correlation and linear regression were used to determine whether ethanol dose (g/kg) consumed and BEC were related for each age group.

Behavioral analyses

Ethanol consumption (g/kg) was analyzed in the 10S10E group using mixed measures ANOVA, with BEC group as the between subjects factor, and session as a within subjects repeated measure. Cumulative ethanol intake prior to PR testing (sessions 1-14; g/kg) was analyzed using one-way ANOVA between BEC group. Behavioral

measures in both PR and "relapse" tests were analyzed using an ANOVA with BEC group, age, and naltrexone dose (0, 0.25, 0.5 mg/kg) as between subject factors. For all analyses, Bonferroni's post hoc comparisons were used as appropriate, and results with $p<0.05$ were assigned significance.

RESULTS

Sweetened ethanol consumption

Sweetened ethanol consumption increased across sessions throughout the experiment for all groups ($F_{13,767}=23.52$, $p<0.001$). Prior to the BEC test, there were no significant differences in ethanol intake across operant sessions between adolescent subgroups, although a trend existed for ND adolescents to consume less ethanol ($F_{1,34}=3.48$, $p=0.07$). When adolescent subgroups were combined, there were no significant differences in ethanol intake across operant sessions prior to BEC test between adolescent and adult rats (Fig. 2.2A operant sessions 1-7; $F_{1,60}=0.74$, ns). However, when ethanol intake was totaled prior to PR test, there were significant differences between adolescent subgroups ($F_{1,34}=11.02$, $p<0.01$). Total ethanol consumption history was significantly different between the 3 groups ($F_{2,59}=6.13$, $p<0.01$). Adolescent rats with nondetectable BECs consumed significantly less total ethanol prior to the PR test (Fig. 2.2B operant sessions 1-14; $p<0.01$).

Blood ethanol concentration (BEC) after operant sweetened ethanol self-administration

Blood samples were taken at three different time points (12, 22, or 35 minutes) after the start of drinking 10S10E in the appetitive/consummatory model, and we previously reported BEC data from the 12- and 22-minute sampling points (Doherty and Gonzales, 2015). Our previous work indicated 35 minutes following the start of drinking was the most appropriate point to measure peak BEC so we used this group for further analyses. For the 35-minute samples, in agreement with our previous findings (Doherty and Gonzales 2015), 58% of adolescent rats had little or no detectable ethanol in their blood (BEC < 5 mg/dl) despite drinking at least 0.3 g/kg ethanol in the operant session. In contrast, all adult rats that consumed at least 0.3 g/kg showed detectable BECs. Ethanol consumption on the BEC test day significantly differed between 3 groups ($F_{2,23}$=18.15, $p<0.001$). ND adolescent rats drank significantly less ethanol (0.8 ± 0.2 g/kg) on BEC test day compared to adult (1.4 ± 0.2 g/kg) and adolescent (1.8 ± 0.6 g/kg) rats with detectable BECs ($p<0.01$). At the 35-minute sampling time, BEC and ethanol dose (g/kg) consumed were significant correlated in both adults and adolescents with detectable BECs (Fig. 2.3; adults, $F_{1,7}$=9.29, $p<0.05$; adolescents, $F_{1,5}$=7.64, $p<0.05$).

Effect of naltrexone on PR schedule of reinforcement

Following two weeks of operant sweetened ethanol self-administration with the appetitive/consummatory model and four days using a FR2 schedule of reinforcement, rats underwent a PR session to test the ability of naltrexone (0, 0.25, 0.5 mg/kg; s.c.) to

reduce ethanol intake and breakpoint value in a PR test. No significant differences were found between the adolescent subgroups on sweetened ethanol consumption ($F_{1,30}$=0.02, ns) or breakpoint value ($F_{1,30}$=0.49, ns) in the PR test. Therefore, age of initiation of operant sweetened ethanol self-administration was used to assess the effects of naltrexone during the PR test. Naltrexone treatment significantly reduced sweetened ethanol intake during the PR session in both adolescent and adults (Fig. 2.4A; $F_{2,54}$=10.70, $p<0.001$). Post hoc tests revealed both doses of naltrexone significantly reduced sweetened ethanol intake ($p<0.01$). Naltrexone also significantly reduced breakpoint values in both age groups (Fig. 2.5A; $F_{2,54}$=4.41, $p<0.05$), with the 0.5 mg/kg dose exhibiting a significant reduction during post hoc analysis ($p<0.05$). In control rats that only drank 2S throughout the experiment, naltrexone or age did not significantly affect sucrose intake (g/kg) during the PR session (Fig. 2.4B; NTX $F_{1,24}$=0.27, ns; age $F_{1,24}$=2.80, ns). Naltrexone also did not affect sucrose breakpoint values in control rats (Fig. 2.5B; NTX $F_{1,24}$=0.04, ns; age $F_{1,24}$=0.04, ns).

Effect of naltrexone on "relapse" of sweetened ethanol or sucrose after abstinence

The efficacy of naltrexone to reduce "relapse" behavior was examined after 13 days of forced abstinence from sweetened ethanol or sucrose. No significant differences were found between adolescent subgroups in total lever presses during the "relapse" test ($F_{1,30}$=1.27, ns). Naltrexone treatment significantly reduced total lever presses during the "relapse" test in both age groups (Fig. 2.6A; $F_{2,56}$=4.48, $p<0.05$), with the 0.5 mg/kg dose exhibiting the most robust reduction during post hoc analysis ($p<0.05$). Interestingly, rats

that began drinking sweetened ethanol in adolescence pressed significantly fewer times regardless of naltrexone treatment (main effect age; $F_{1,56}=4.85$, $p<0.05$). In control rats with a history of drinking 2S, naltrexone did not affect sucrose lever presses (Fig. 2.6B; $F_{1,23}=0.33$, ns) although a trend existed for the adolescent group to press more times than adults ($F_{1,23}=3.37$, $p=0.08$)

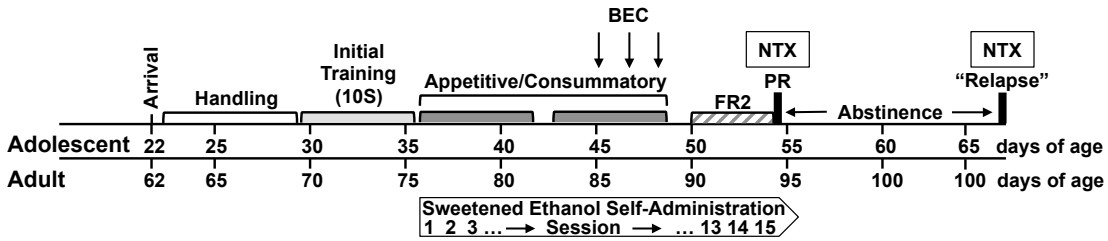

Figure 2.1 Timeline of operant sweetened ethanol self-administration in adolescent and adult rats

Adolescents arrived at postnatal day (P) 22, adults ~P62. All rats were initially trained on a 10% sucrose solution (10S). Sweetened ethanol (EtOH) (10% sucrose + 10% EtOH [10S10E]) self-administration began at P36 for adolescents, ~P76 for adults. Rats were tested on appetitive/consummatory, fixed ratio 2 (FR2), progressive ratio (PR), and "relapse" models of operant self-administration. Blood EtOH concentration (BEC) was measured across the last three days of appetitive/consummatory sessions. Rats received naltrexone ([NTX] 0, 0.25, 0.5 mg/kg; subcutaneous) 30 minutes prior to PR and "relapse" sessions. Control rats drank 2% sucrose (2S) throughout the experiment. Figure adapted from Doherty and Gonzales, 2015.

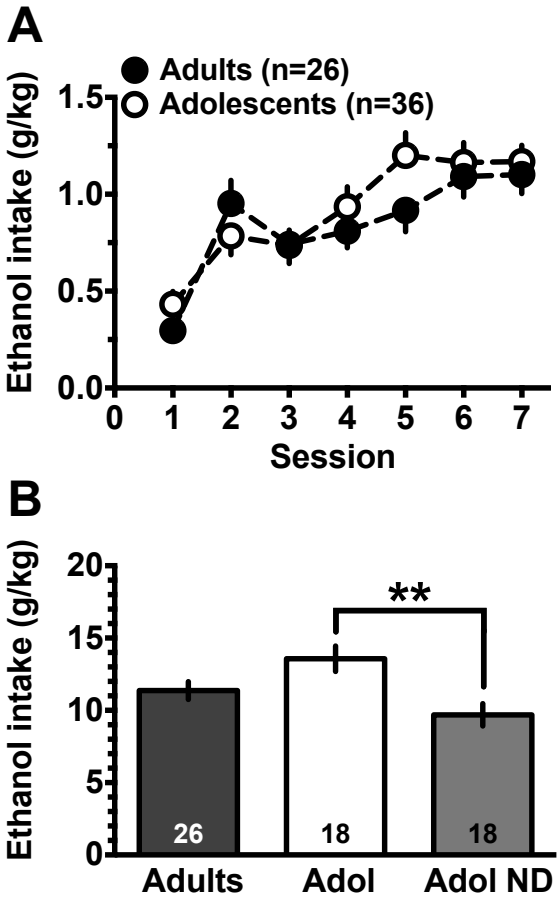

Figure 2.2 Amount of sweetened ethanol consumed prior to the blood ethanol concentration test and total ethanol consumed (g/kg) prior to the progressive ratio test

(A) Sweetened ethanol intake prior to blood ethanol concentration (BEC) test (sessions 1-7) did not differ between age groups. (B) When ethanol intake was totaled from all operant models prior to progressive ration (PR) test (sessions 1-14), adolescents with no detectable BEC (Adol ND) drank significantly less sweetened ethanol than adolescents with detectable BECs (** indicates $p<0.01$). For both panels data presented as mean ± sem, and group n is shown within bars.

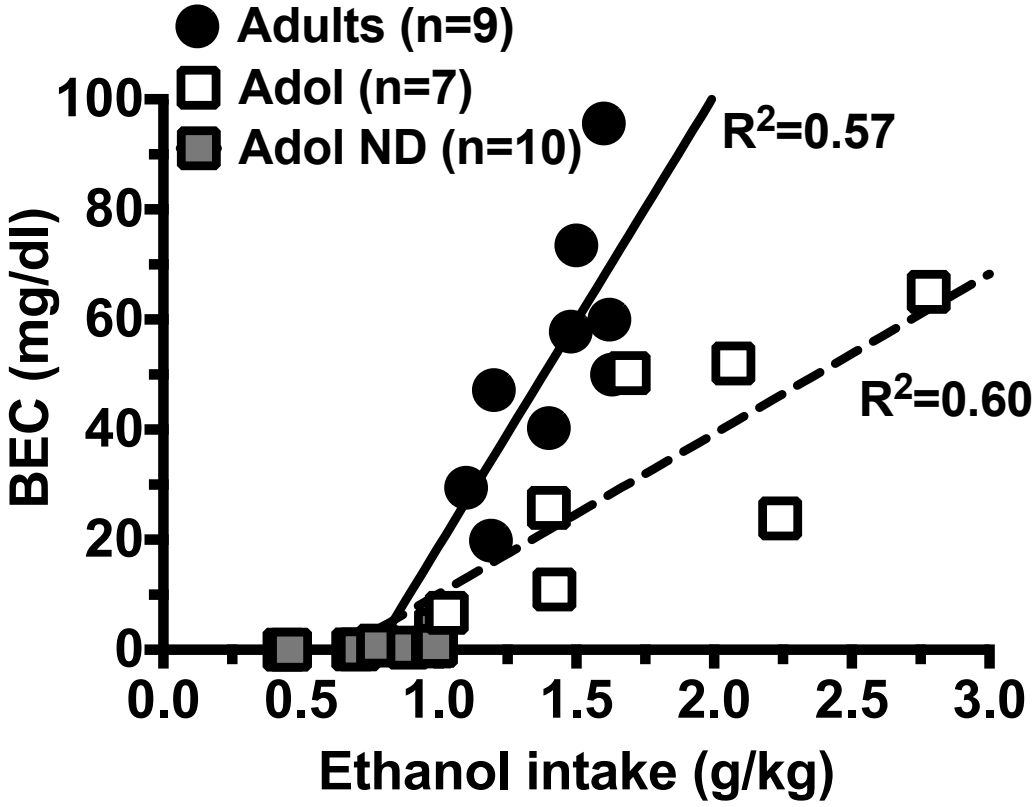

Figure 2.3 Blood ethanol concentration (BEC) in adolescents and adults 35 minutes after the start of drinking sweetened ethanol in an operant self-administration session

A large proportion of adolescents had little or no detectable ethanol (EtOH) in their blood after drinking sweetened EtOH (nondetectable (ND) adolescent; BEC < 5 mg/dl; grey squares). In contrast, all of the adults (black circles, solid line), and half of adolescents (open squares, dotted line) had BEC > 5 mg/dl, and their BEC significantly correlated with EtOH intake (adolescent $p<0.05$; adult $p<0.05$).

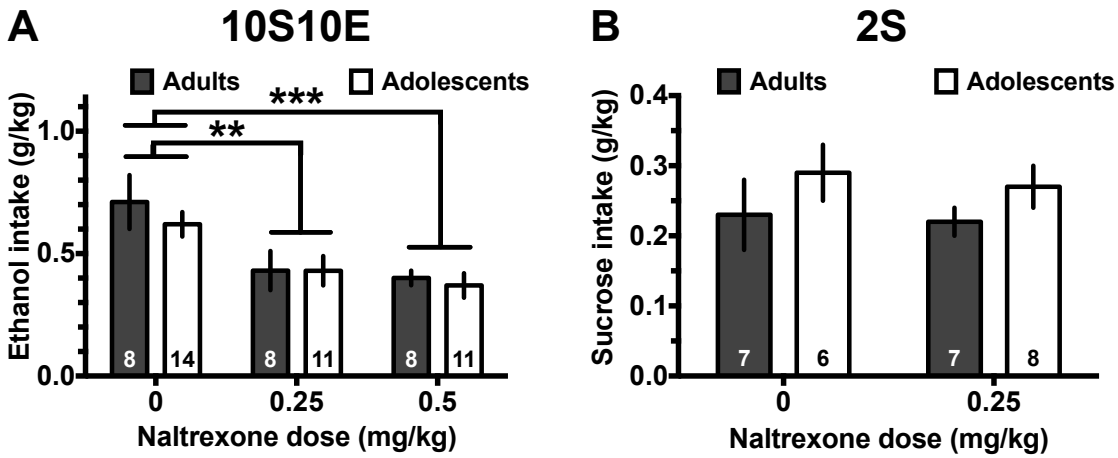

Figure 2.4 Naltrexone treatment on sweetened ethanol or sucrose intake during the progressive ratio test

(A) Naltrexone significantly reduced sweetened ethanol (EtOH) consumption in the progressive ratio (PR) test in adolescents and adults (** indicates $p<0.01$, *** indicates $p<0.001$). 10S10E=10% sucrose + 10% ethanol. (B) Naltrexone did not alter sucrose consumption during the PR test in control rats. 2S=2% sucrose. For both panels data presented as mean ± sem, and group n is shown within bars.

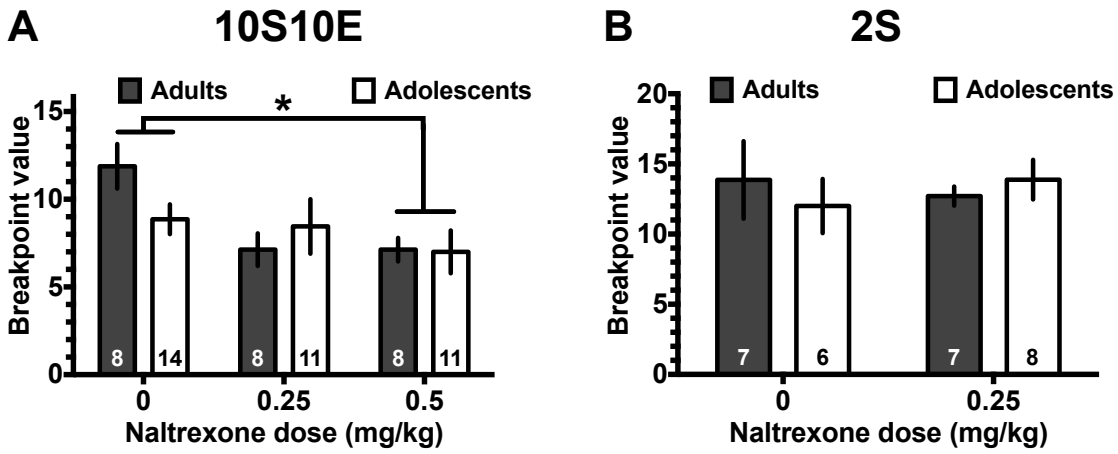

Figure 2.5 Naltrexone treatment on progressive ratio (PR) breakpoint values in sweetened ethanol or control sucrose groups

(A) Naltrexone significantly reduced breakpoint values of adolescents and adults drinking sweetened ethanol during the PR test (* indicates $p<0.05$). 10S10E=10% sucrose + 10% ethanol. (B) Naltrexone did not alter breakpoint values of control sucrose groups during the PR test. 2S=2% sucrose. For both panels data presented as mean ± sem, and group n is shown within bars.

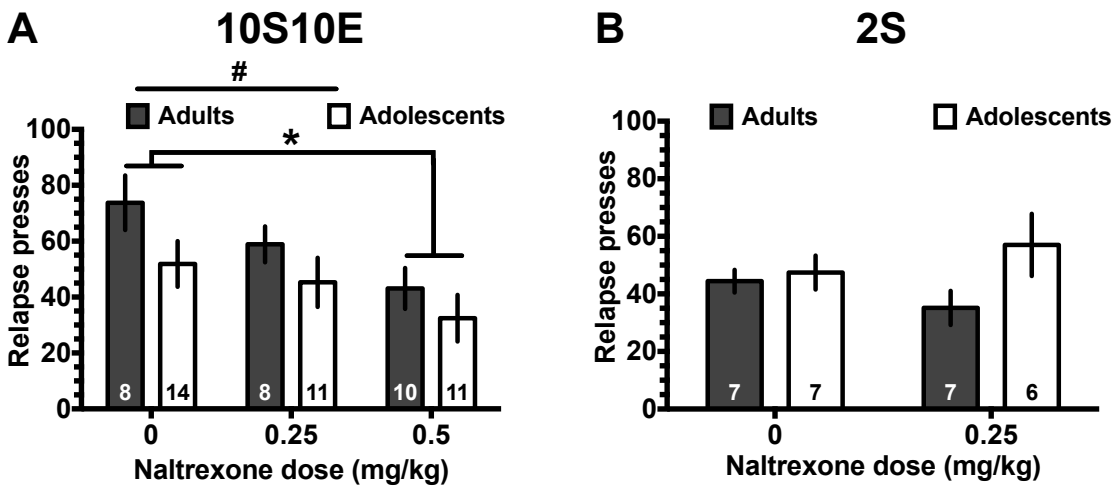

Figure 2.6 Naltrexone treatment on "relapse" of ethanol or sucrose seeking after a period of abstinence

(A) Naltrexone significantly reduced "relapse" of 10S10E-paired lever presses in adolescents and adults with a history of drinking sweetened ethanol (* indicates $p<0.05$). Rats that began drinking sweetened ethanol in adolescence pressed significantly fewer times across all doses compared to rats that began drinking in adulthood (# indicates $p<0.05$). 10S10E=10% sucrose + 10% ethanol. (B) Naltrexone did not alter "relapse" of 2S-paired lever presses in control group with a history of drinking sucrose. 2S=2% sucrose. For both panels data presented as mean ± sem, and group n is shown within bars.

DISCUSSION

The results of this study are the first evidence to indicate that systemic naltrexone is effective in reducing operant sweetened ethanol consumption, motivation to obtain sweetened ethanol and "relapse" behavior in rats that began operant sweetened ethanol self-administration in adolescence. Naltrexone significantly reduced ethanol intake and breakpoint value in adolescents during the PR test. In the "relapse" test following 13 days of forced abstinence, naltrexone also significantly reduced lever presses for ethanol in rats that began operant sweetened ethanol self-administration in adolescence. Interestingly, age of onset of ethanol self-administration may influence expression of "relapse" behavior as rats that began operant sweetened ethanol self-administration in adolescence pressed fewer times in the "relapse" test compared to adults. As a positive control, naltrexone also significantly reduced ethanol intake, breakpoint and "relapse" behavior in rats that began operant sweetened ethanol self-administration in adulthood. All of the reported effects of naltrexone were specific to sweetened ethanol, as naltrexone did not affect sucrose consumption, motivation to obtain sucrose or "relapse" behavior in control rats.

The present study is the first to show that naltrexone reduces sweetened ethanol intake and motivation to obtain sweetened ethanol using a PR schedule of reinforcement in rats that began sweetened ethanol operant self-administration in adolescence. Here we extend published findings in adult rats that naltrexone significantly decreases operant ethanol self-administration and breakpoint value (Ciccocioppo *et al*. 2003; Gonzales and Weiss 1998; Hay *et al*. 2013; Henderson-Redmond and Czachowski 2014) to now

include similar findings in adolescent rats. Naltrexone has previously been shown to reduce ethanol consumption in adolescent alcohol-preferring (P) rats using a two-bottle choice paradigm (Sable *et al*. 2006). Our data extend these findings to indicate that naltrexone is effective at reducing sweetened ethanol intake in adolescents at similar levels to adult rats using an operant self-administration model, known to have high predictive validity for testing treatment efficacy (Carter and Griffiths 2009). Importantly, in our study rats that began operant sweetened ethanol self-administration in adolescence were 54 days old during the PR test. Some studies suggest the range of 28-42 days as an estimate of "adolescence" in rodents (Spear 2000). However, many neural circuits, including midbrain dopamine neurons that are implicated in ethanol-related behaviors, are still undergoing maturation past this age range (McCutcheon and Marinelli 2009).

The mechanisms by which naltrexone suppresses ethanol self-administration behavior has been suggested to involve antagonism of opioid receptors in adult models (Gonzales and Weiss 1998; Herz 1997). Specifically, the mu opioid receptor has been linked to motivation to consume ethanol in adult rodent models (Herz 1997; Richard and Fields 2016). Limited evidence suggests that opioid receptor signaling is present in relevant brain areas in adolescents similar to that in adults (Ellgren *et al*. 2008; Palm and Nylander 2014). Therefore, it is likely that the reduction of operant ethanol self-administration in adolescents and young adults that we report here is due to opioid receptor antagonism similar to the hypothesized mechanism for adults. Opioid antagonists have also been shown to decrease ethanol intake in preweanling rats (Chotro and Arias 2003; Hallmark and Hunt 2004). However, the precise mechanisms underlying

the reduction in operant ethanol self-administration and breakpoint value by systemic naltrexone in adolescence remain unknown.

Our study also provides the first evidence that naltrexone significantly reduces lever presses for sweetened ethanol in an operant "relapse" test in rats that began drinking in adolescence. Systemic naltrexone has previously been shown to decrease cue- and context-induced reinstatement for ethanol seeking following extinction in adult rats (Burattini *et al*. 2006; Ciccocioppo *et al*. 2003; Katner *et al*. 1999). We did not extinguish operant responding in this study but instead exposed rats to 13 days of forced abstinence in their home cage prior to our model of "relapse" behavior. This model of ethanol seeking behavior simulates aspects of alcohol relapse in humans as many people undergo forced periods of abstinence (e.g., hospitalization, incarceration) prior to being re-exposed to alcohol-related cues that may precipitate relapse (reviewed in Reichel and Bevins 2009). Recently, alcohol craving was shown to increase over time during a 60-day period of forced abstinence in human alcoholics (Li *et al*. 2015). Future studies should investigate whether naltrexone reduces "relapse" lever pressing for ethanol following longer periods of forced abstinence and if naltrexone efficacy changes across an "incubation of craving" period.

An unexpected finding from our study is that age of onset of operant sweetened ethanol self-administration affected "relapse" behavior differently when tested in adulthood. Rats that began sweetened ethanol self-administration in adolescence pressed significantly fewer times in the "relapse" test of seeking behavior than rats that began in adulthood. Attenuated cue-induced reinstatement has previously been shown in

adolescent rats compared to adults following operant self-administration of cocaine, heroin and morphine (Doherty *et al*. 2009; Doherty and Frantz 2012; Li and Frantz 2009). Therefore, it is possible a common mechanism exists across drugs of abuse that leads to reduced "relapse" behavior in adulthood specific to onset of operant self-administration that begins in adolescence. Our previous work did not report age differences in "relapse" lever presses using a behavioral protocol that differed slightly from that used in the present study. In the previous work, the "relapse" test occurred without injections because we did not test whether any pharmacological agents would alter the behavior (Doherty and Gonzales 2015). Therefore, an alternative explanation for the age difference seen in the current work is the stress from injection may have affected the two age groups differently. However, adolescent drug or alcohol exposure has been shown to increase stress-induced reinstatement or consumption compared to adults (Siegmund *et al*. 2005; Wong and Marinelli 2016). As such, it is unlikely injection stress is the cause of reduced "relapse" behavior in the rats that began operant self-administration in adolescence. Additional work is needed to determine the possible mechanisms that underlie the age differences in relapse behavior we observed here.

A significant proportion of the adolescent rats had little or no detectable BEC on the test day (sessions 8-10) within the appetitive/consummatory phase of the experiment. We previously reported that following an intragastric challenge of 1 g/kg sweetened ethanol, adolescent rats showed significantly lower peak BEC, shorter time to BEC peak and a trend for faster elimination rate suggesting a larger volume of distribution and higher metabolism compared to adults (Doherty and Gonzales 2015). The ND adolescent

rats in the current study also consumed significantly less ethanol on BEC test day. Therefore, it is likely the nondetectable BECs may be attributed to the lower dose of ethanol consumed along with the aforementioned pharmacokinetic differences in adolescent rats. Despite ND adolescents consuming significantly less total ethanol prior to the PR test, these rats did not differ from adolescents with detectable BECs in PR and "relapse" tests. Together, these results suggest the ND adolescents experienced the central pharmacological effects of ethanol prior to the PR and "relapse" tests. One limitation to our interpretation is that BEC was only tested on one day across the experiment. Future studies are necessary to fully characterize the pharmacokinetics of operant sweetened ethanol self-administration throughout the entire period of adolescence to adulthood.

Collectively, our results suggest naltrexone is effective at reducing ethanol consumption, motivation to obtain ethanol and "relapse" of lever pressing behavior in rats that began operant sweetened ethanol self-administration in adolescence or adulthood. Recent clinical research has demonstrated naltrexone can reduce heavy drinking and blunt craving in adolescents (ages 15-19) and young adults (ages 18-25), although larger trials are needed (Miranda *et al*. 2014; O'Malley *et al*. 2015). The current findings provide insight into consummatory, motivational and "relapse" behaviors that may be targeted by naltrexone in an adolescent animal model of moderate operant sweetened ethanol consumption, and highlight the need for continuing clinical investigation of efficacy in this population. Together, these results support the use of

opioid receptor antagonism as a potential mechanism for effective treatment for late adolescents and young adults to reduce alcohol consumption.

Chapter 3: High sensitivity HPLC method for analysis of *in vivo* extracellular GABA using optimized fluorescence parameters for *o*-phthalaldehyde (OPA)/sulfite derivatives

ABSTRACT

Reversed-phase HPLC with derivatization using *o*-phthalaldehyde (OPA) and sulfite allows electrochemical detection of γ-aminobutyric acid (GABA) in microdialysis samples. However, OPA/sulfite derivatives have been reported to produce a lower fluorescent yield than other OPA/thiol derivatives. To overcome this limitation we examined excitation and emission spectra, reaction time, pH, and concentration of reagents in the derivatization solution. Optimal detection parameters were determined as λ_{ex}=220 nm and λ_{em}=385 nm for maximal fluorescence. The derivatization reaction occurred immediately and the product was stable up to 10 minutes. A pH of 10.4 for the borate buffer used in the derivatization solution was significantly better than lower pH. Increasing the amount of sulfite combined with diluting the derivatization solution in borate buffer resulted in complete separation of the GABA peak from contaminants without any loss in signal. Controlling the temperature of the detector at 15°C significantly improved sensitivity with a detection limit of approximately 1 nM. To validate this assay, we performed microdialysis in the dorsal striatum and ventral tegmental area (VTA) of adult Long Evans rats. GABA concentrations in dialysates were

determined using external standards and standard additions, in order to further confirm interfering peaks were not present in biological samples. Within the dorsal striatum (n=4), basal GABA concentrations were 12.9±2.2 and 14.5±2.2 nM (external and additions, respectively). Respective basal GABA concentrations in the VTA (n=3) were 4.6±1.1 and 5.1±0.6 nM. Thus, we have developed a novel, sensitive fluorescence method to determine GABA in microdialysates using HPLC of an OPA/sulfite derivative.

INTRODUCTION

γ-aminobutyric acid (GABA) is the primary amino acid neurotransmitter involved in inhibitory synaptic transmission and alterations in GABAergic signaling contribute to many neurological conditions including epilepsy, schizophrenia and anxiety disorders (Wong *et al*. 2003). Microdialysis is a technique commonly used in basic and clinical neuroscience to measure the concentration of GABA in the extracellular space (Chefer *et al*. 2009b; Shah *et al*. 2002). Many separation approaches exist for analysis of amino acid neurotransmitters, although high performance liquid chromatography (HPLC) remains one of the most widely used in neuroscience (Shah *et al*. 2002). However, GABA is neither fluorescent nor electroactive and requires a derivatization procedure prior to detection with these methods (Roth 1971; Shah *et al*. 2002).

One of the most well characterized derivatization reagents used for analysis of amino acid neurotransmitters is *o*-phthalaldehyde (OPA), which reacts with amines in the presence of a nucleophile to form electroactive and/or fluorescent isoindole derivatives (Chen *et al*. 1979; Lindroth and Mopper 1979; Roth 1971; Simons and Johnson 1978). The use of OPA in conjunction with thiols (e.g. 2-mercaptoethanol (MCE), 3-mercaptopropionic acid (MPA)) has been widely used for both electrochemical (ECD) or fluorescence detection (FLD) of low concentrations of GABA in microdialysis samples (Bourdelais and Kalivas 1991; Donzanti and Yamamoto 1988; Durkin *et al*. 1988; Piepponen and Skujins 2001; Rea *et al*. 2005; Westerink and de Vries 1989; Zhang *et al*. 2005). However, in addition to a pungent odor, thiol derivatives can be unstable (Lasley *et al*. 1984; Lindroth and Mopper 1979). This can be particularly problematic when

quantifying GABA in microdialysis samples due to highly specific chromatographic conditions required for separation (Rea *et al*. 2005).

An alternative to thiols is using OPA in the presence of a sulfite group as the nucleophile to form an N-alkyl-1-isoindole sulfonate derivative that has been reported to be more stable than thiol-formed derivatives (Jacobs 1987; Rowley *et al*. 1995). OPA/sulfite derivatization has been successfully used in multiple applications to measure GABA in brain microdialysates using ECD (Bongiovanni *et al*. 2001; Reinhoud *et al*. 2013; Rowley *et al*. 1995; Smith and Sharp 1994). These ECD methods require high working potentials (0.7-0.85 V) for GABA detection and can result in more frequent maintenance of the electrochemical cell. In contrast, FLD offers advantages in ease of operation and stability over long periods of time. However, OPA/sulfite derivatives were reported to be significantly less fluorescent than OPA/thiol derivatives and not useful for high sensitivity analysis of GABA in microdialysis samples (Kehr 1993).

Previous studies using FLD quantified GABA in tissue or brain microdialysates using OPA/thiol derivatives reported ranges of 330-365 nm and 420-530 nm for excitation (λ_{ex}) and emission (λ_{em}) wavelengths, respectively (deFreitas Silva *et al*. 2009; Kehr 1998; Peng *et al*. 2008; Vihavainen *et al*. 2008). Recently, excitation and emission spectra were thoroughly examined for amino acids derivatized with OPA/MPA, including GABA. These authors observed the maximal signal for the OPA/MPA derivative of GABA occurred at λ_{ex}=229 nm and λ_{em} =450 nm, which resulted in a six-fold greater signal compared to previous wavelengths used (Perucho *et al*. 2015). Therefore, we

hypothesized the low fluorescence yield previously reported for OPA/sulfite derivatives may have been due to suboptimal detection parameters.

Thus, the aim of the present study was to develop a sensitive method for quantification of GABA in brain microdialysis samples using HPLC-FLD of an OPA/sulfite derivative. We determined optimal detection wavelengths, derivatization components, and reaction procedures for analysis of GABA. Additionally, we used *in vivo* microdialysis to quantify extracellular GABA in the dorsal striatum or ventral tegmental area (VTA) of Long Evans rats using external standards and standard additions to validate our method.

MATERIALS AND METHODS

Reagents

GABA, GABase from Pseudomonas fluorescens, OPA, sodium dihydrogen phosphate dihydrate, sodium sulfite and sodium tetraborate decahydrate were obtained from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). Methanol was purchased from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ, USA) and absolute ethanol from AAPER (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA). Artificial cerebral spinal fluid (ACSF) for microdialysis experiments consisted of 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.4 mM D-glucose. All solutions were made with

deionized water obtained from a Milli-Q system (Millipore, Billerica, MA, USA) and filtered using 0.2 μm nylon filters (Pall Corp., Ann Arbor, MI, USA).

Instrumentation

Liquid chromatography

HPLC separation was achieved using a Luna C18(2) column (150 x 1.0 mm, 3 μm; Phenomenex, Torrance, CA, USA), Antec LC110 pump (Antec Leyden, Zoeterwoude, Netherlands), in-line degasser, column compartment (maintained at 40°C) equipped with a manual injector (9725i Rheodyne, Cotati, CA, USA) and polyetheretherketone (PEEK) 20 μl injection sample loop. Mobile phase was pumped at 0.05-0.1 ml/min. Injection volume varied from 5 to 10 μl using Hamilton syringes (1702 or 705, Hamilton Co., Reno, NV, USA).

Mobile phase

The mobile phase consisted of 0.1 M sodium dihydrogen phosphate dihydrate ($NaH_2PO4 \cdot 2H_2O$) adjusted to pH 4.5 using 1 M phosphoric acid and 10-18% (v/v) methanol added to optimize chromatography.

Fluorescence detection

Analyte detection was achieved using a Jasco FP-4020 fluorescence detector with a 12.7 μl analytical flow cell (Jasco Corp., Tokyo, Japan). The detector gain was set to 1000, and the response time was 3 seconds. We attached a glass coverslip to the flow cell

to serve as an emission filter. Slit widths of 20 nm for excitation and 40 nm for emission were used.

Optimal wavelengths were determined by performing excitation and emission scans using the Jasco FP-4020. Differences in signal of the GABA derivatization product (1 μM) and the mobile phase were calculated for corresponding scans. Scans were triggered manually after the peak passed a threshold point of half the total signal of the GABA peak, and were taken at a scan speed of 200 nm/sec. Scans were performed at a gain set at 100 to ensure outputs were on scale.

Chromeleon 6.8 Chromatography Data System software (Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition and analysis. All experiments were run at minimum in triplicate across days. Chromatographic peaks were required to have a signal to background noise ratio of at least 3:1 for analysis.

Derivatization procedure

The derivatization working solution was made by dissolving 11 mg *o*-phthaldialdehyde (OPA) in 250 μl absolute ethanol, 250 μl 1 M sodium sulfite (sulfite; Na_2SO_3) and 4.5 ml 0.1 M sodium tetraborate decahydrate (borate; $Na_2B_4O_7 \cdot 10H_2O$; adjusted to pH 10.4 with 5 M NaOH) according to the methods of Smith and Sharp (Smith and Sharp 1994). During the optimization experiments, final volumes of sulfite and borate buffer used in working solution were adjusted to improve chromatography (detailed in Section 3.3). The sulfite solution was made every 2 days and the borate solution made every 7 days to achieve optimal signal. Both solutions were stored in glass

at room temperature. The OPA/sulfite working solution was stored in covered plastic vials at 4°C up to 24 hours. Stock GABA standards were prepared by dissolving GABA in deionized water at a concentration of 1 mM and stored at 4°C for up to 1 month. Standard dilutions were made fresh daily in either water or borate buffer based on the experiment. Polypropylene centrifuge tubes were used for standard and sample tubes to reduce loss of GABA by adsorption (Boyd and Kennedy 1998; Rowley *et al*. 1995).

The derivatization procedure consisted of combining 9 µl of GABA standard or sample with 2 µl borate and then adding 0.5 µl OPA working solution. The additional borate added to the reaction mixture was used due to more reproducible separation. This reaction mixture was manually mixed with a pipette and incubated at room temperature in darkness.

In vivo microdialysis procedures

Adult, male Long Evans rats (n=7; 290-301 g upon arrival; Charles River Laboratories, Raleigh, NC, USA) were surgically implanted with a 21 gauge guide cannula (Plastics One, Roanoke, VA, USA) above the dorsal striatum (in mm relative to bregma and skull surface: 0.0 antero-posterior, +3.7 lateral, -3.0 ventral) or VTA (-5.8 antero-posterior, +2.1 lateral, -4.6 ventral, angled 10° toward midline) using procedures similar to our previous studies (Doyon *et al*. 2005). The dorsoventral coordinate represents the bottom of the guide cannula, and the probes for either brain region extend 4.0 mm below the cannula when seated into the guide. Animals were allowed at least 5 days of recovery prior to microdialysis experiments.

Microdialysis probes (length of probe active area: dorsal striatum 3 mm, VTA 1 mm; 13,000 molecular weight cutoff) were constructed similar to Pettit and Justice (Pettit and Justice 1991a) and implanted 12-18 hours prior to experiments. Probes were perfused with ACSF at 0.2 μl/min flow rate overnight, and then increased to 1.0 (VTA) or 2.0 μl/min (dorsal striatum) at least two hours prior to dialysis sampling. Microdialysis sampling occurred during 0800 – 1600, during the light cycle of the animals. Dialysis samples were immediately frozen on dry ice and stored at -80ºC until analysis. Microdialysis probe placements were confirmed via histological analysis. All animal procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals (8th Ed., 2011) and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Standard additions

Basal dialysates were pooled for each rat. Each pooled sample was analyzed in triplicate. Pooled dialysates from dorsal striatum were diluted 1:1 in borate prior to derivatization to reduce large peaks of biological origin present in the samples. The first injection used 9 µl of the diluted dialysate sample mixed with 2 µl borate and 0.5 µl of the optimal OPA working solution determined in Section 3.3. The two remaining injections substituted the 2 µl borate in the derivatization with an equal volume of GABA standard made in borate. GABA concentration used in standard additions varied based on brain region (dorsal striatum: 25, 50 nM; VTA: 10, 25 nM). Basal values are not corrected for probe recovery.

Statistics

GABA signals in optimization experiments were analyzed using analysis of variance (ANOVA) or t-tests. Basal GABA concentrations calculated using external standards and standard additions were compared using paired t-tests. Bonferroni's post hoc comparisons were used as appropriate, and results with $p<0.05$ were assigned significance; ns=not significant. Data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Optimization of excitation and emission wavelengths

The fluorescence of thiol-substituted isoindoles has a range of reported excitation and emission wavelengths used for detection of OPA derivatives of GABA (Perucho *et al*. 2015). Using a 5-minute precolumn reaction time and the derivatization solution described in Section 2.3, we initially compared excitation and emission conditions similar to previous reports using OPA/MCE or MPA derivatives (λ_{ex}=330 nm, λ_{em}=450 nm) (Rea *et al*. 2005; de Freitas Silva *et al*. 2009) with conditions recently described to be more sensitive for OPA/MPA GABA derivatives (λ_{ex}=240, λ_{em}=450 nm) (Perucho *et al*. 2015). We observed a significant increase in GABA signal at 240 nm for the excitation wavelength (data not shown). Following a series of manual adjustments, we performed the excitation spectrum scan between 200 and 360 nm with the emission set at 400 nm.

The peak of maximal intensity was λ_{ex}=220 nm, with another significantly smaller peak at 330 nm. Then, we performed the emission spectrum scan between 330 and 600 nm with the excitation set at 220 nm. The peak of maximal intensity was λ_{em}=385 nm. Scans were repeated in triplicate using derivatization solution determined in Section 3.3 to confirm results. Optimal wavelengths to achieve the highest signal for an OPA/sulfite GABA derivative were determined to be excitation λ_{max}=220 nm and emission λ_{max}=385 nm (Fig. 3.1). However, the emission wavelength was changed to 400 nm in order to optimize chromatography. We used λ_{ex}=220 nm and λ_{em}=400 nm for experiments unless otherwise noted.

Derivative stability

Initially using λ_{ex}=229 nm and λ_{em}=425 nm for detection, the stability of the OPA/sulfite GABA derivative was tested by comparing the signal after different reaction times for the derivatization procedure up to 10 minutes. The zero time point was mixed and immediately manually injected into the system, which took approximately 30 seconds total. We found no significant differences in signal achieved between time points up to 10 minutes (Fig. 3.2; $F_{3,8}$=2.89, ns). Therefore, the GABA OPA/sulfite derivative appears to display maximal fluorescence immediately under our conditions. This is in contrast to Rowley et al. who reported maximal GABA signal at 5 minutes of an OPA/sulfite derivative using ECD (Rowley *et al*. 1995). Additional reaction times were not tested, therefore it is possible the derivative is stable longer than 10 minutes. The

reaction was standardized to 1 minute in darkness prior to manual injection for all following experiments.

Optimization of derivatization procedures

Effect of pH

Derivatization conditions including reagent composition and ratio of OPA to the nucleophile additive are well documented to significantly influence chromatography for other thiols reviewed in (Molnár-Perl and Bozor 1998). In order to determine the optimal OPA/sulfite derivatization conditions using fluorescence detection (λ_{ex}=229 nm and λ_{em}=425 nm), we first examined the effect of pH of borate buffer (8.4, 9.4, 10.4) on the GABA derivative signal. For each pH tested, the borate buffer solution used in the OPA derivatization working solution was made in triplicate. There was a significant difference in GABA signal detected between pH of borate buffers tested ($F_{2,6}$=471.2, $p<0.001$). Post hoc analyses revealed no significant differences between pH 10.4 and 9.4, however at pH 8.4 the GABA signal was significantly lower (Fig. 3.3; $p<0.001$). Therefore, for all additional experiments the pH of the borate buffer used was adjusted to 10.4.

The stock derivatization working solution was made by dissolving 11 mg OPA in 250 µl absolute ethanol, 250 µl 1 M sulfite and 4.5 ml 0.1 M borate based on procedures reported for electrochemical detection of OPA/sulfite derivatives (Smith and Sharp 1994). Using this working solution, another peak following GABA sometimes interfered with quantification of GABA concentration. Adjusting the methanol concentration in the mobile phase improved separation of GABA from this unknown peak (data not shown),

however relying on these adjustments alone reduced reproducibility over time due to the extreme sensitivity of GABA to methanol (Rea *et al*. 2005). As a result, we sought to optimize the derivatization working solution to minimize excess OPA and therefore reduce other derivative products leading to an improvement in the separation of the GABA peak.

Effect of OPA:sulfite ratio

We first investigated how the mole ratio of OPA to sulfite in the derivatization solution would influence chromatography. The mole ratio in the stock derivatization solution was 1 OPA to 3 sulfite (1 O: 3 S) as originally reported by Smith and Sharp (Smith and Sharp 1994). We increased the amount of sulfite in the derivatization so the mole ratio was 1 OPA to 10 sulfite (1 O: 10 S; high sulfite) to test whether this modification would reduce the peak following GABA in the chromatogram (λ_{ex}=229 nm and λ_{em}=425 nm). This high sulfite derivatization solution was made by dissolving 11 mg *o*-phthaldialdehyde (OPA) in 250 µl absolute ethanol, 820 µl 1 M sulfite and 3.93 ml 0.1 M borate. Both OPA solutions were also diluted 1:10 in borate in an effort to reduce additional peaks in the chromatogram. There was no significant difference in GABA signals observed using the various derivatization solutions (Fig. 3.4; $F_{3,8}$=0.80, ns) and separation of GABA from the trailing contaminant improved significantly using the high sulfite solution.

Effect of OPA concentration

We next investigated whether diluting the OPA working solution with borate buffer would improve chromatography (λ_{ex}=229 nm and λ_{em}=425 nm). We initially compared the stock derivatization working solution with 1:10, 1:30 and 1:100 dilutions in borate and observed a significant difference in signal ($F_{3,8}$=24.97, $p<0.001$). Post hoc comparisons showed only the 1:100 dilution resulted in a significantly lower GABA signal (Fig. 3.5A; $p<0.01$). The other dilutions in borate (1:10 and 1:30) no had effect on GABA signal but did reduce additional peaks in the chromatogram. Using the high sulfite derivatization working solution shown to optimize separation in Figure 4, we tested whether further borate dilution of this solution would improve chromatography now using λ_{ex}=220 nm and λ_{em}=400 nm for detection. Dilutions of the high sulfite derivatization solution in borate resulted in optimal GABA separation with no reduction in signal (Fig. 3.5B; t(10)=0.45, ns). The reduction in additional peaks and improvement in GABA separation between using the original OPA derivatization solution and the OPA solution with higher sulfite diluted in borate buffer is illustrated in Figure 3.6.

Our results show the derivatization protocol determined to optimize GABA sensitivity and resolution was prepared by dissolving 11 mg OPA in 250 µl absolute ethanol, 820 µl 1 M sulfite and 3.93 ml 0.1 M borate, then diluting this OPA working solution 1:20 (v/v) in borate. This derivatization solution was used to further determine method sensitivity and quantify GABA concentration in microdialysis samples using λ_{ex}=220 nm and λ_{em}=400 nm as detection wavelengths.

Effect of temperature on fluorescence

Increases in temperature can reduce fluorescence intensity (Bowen and West 1955; Suzuki *et al*. 1977), which can occur by changes in ambient room temperature or directly from equipment. We tested the sensitivity of our method with no temperature control and using the FLD to maintain temperature at 15°C. The temperature of the column compartment was 40°C for both conditions. When FLD temperature was controlled at 15°C, there was a significant increase in slope of the standard calibration curve (6.25 – 25 nM GABA) reflecting an improvement in the sensitivity of our method (Fig. 3.7; $F_{1,20}$=36.26, $p<0.001$). We further confirmed the increase in sensitivity using standard calibration curves with low concentration GABA standards (1.5 – 12.5 nM). A detection limit of 1 nM was calculated from the average of triplicate calibration curves as the concentration resulting in a signal 3 times the peak-to-peak noise of the baseline from the average of triplicate OPA derivative blanks.

***In vivo* validation using standard additions**

Reported extracellular GABA concentrations from rodent microdialysis studies vary substantially both between and within different brain regions (Fliegel *et al*. 2013). Methodology used in the microdialysis experiments likely contributes to these differences, however chromatographic conditions are also a factor as other peaks of biological origin can interfere with accurate GABA quantification (Rea *et al*. 2005). We applied our method to quantify basal extracellular GABA from microdialysis samples in dorsal striatum and VTA of Long Evans rats. In order to examine whether other peaks

were interfering with the GABA peak in dialysate samples, we used both external standards and standard additions to determine GABA concentrations. Calibration curves from standard additions to pooled dialysate samples displayed good linearity (Fig. 3.8; dorsal striatum R^2=0.97, VTA R^2=1.00). The GABA concentration in samples calculated using external standards was compared to concentration determined from standard additions (x-intercept of the regression) for each rat individually. Average GABA concentrations are shown in Table 3.1. There were no significant differences between basal concentrations calculated using external standards compared to standard additions for either brain region (dorsal striatum: t(3)=2.22, ns; VTA: t(2)=0.91, ns). Our results confirm additional peaks of biological origin are not present underneath the GABA peak. In addition, we further validated the identity of the GABA peak qualitatively using enzyme degradation with GABase from Pseudomonas fluorescens in both standards and dialysate samples (data not shown).

Brain region	**External standards**	**Standard additions**
Dorsal striatum	12.9 ± 2.2 nM	14.5 ± 2.2 nM
VTA	4.6 ± 1.1 nM	5.1 ± 0.6 nM

Table 3.1 Basal GABA concentrations calculated using external GABA standards and standard additions

Dorsal striatum (n=4) and ventral tegmental area (VTA; n=3). Values for dorsal striatum corrected for dilution factor in borate. Basal values are not corrected for probe recovery. Data are presented as mean ± sem.

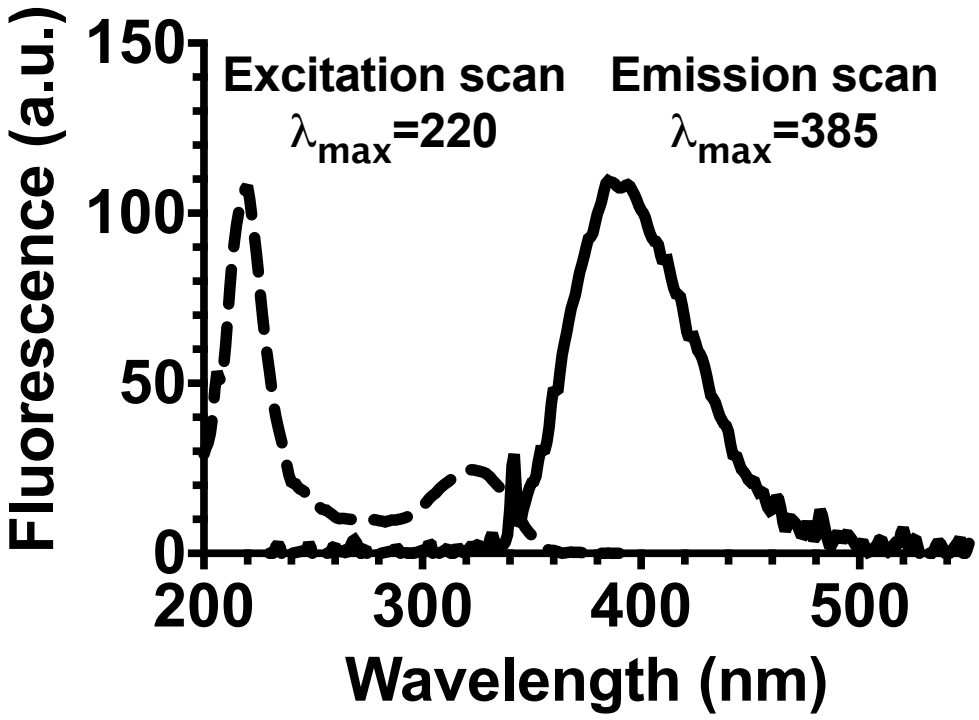

Figure 3.1 Maximal excitation and emission spectra for OPA/sulfite GABA derivative

The difference in signal of the GABA derivatization product and the mobile phase was calculated for each scan and data are presented as mean of three separate scans. Emission wavelength set to 400 nm for excitation scan. Excitation wavelength set to 220 nm for emission scan. λ_{max}=wavelength at maximal signal, a.u.=arbitrary units.

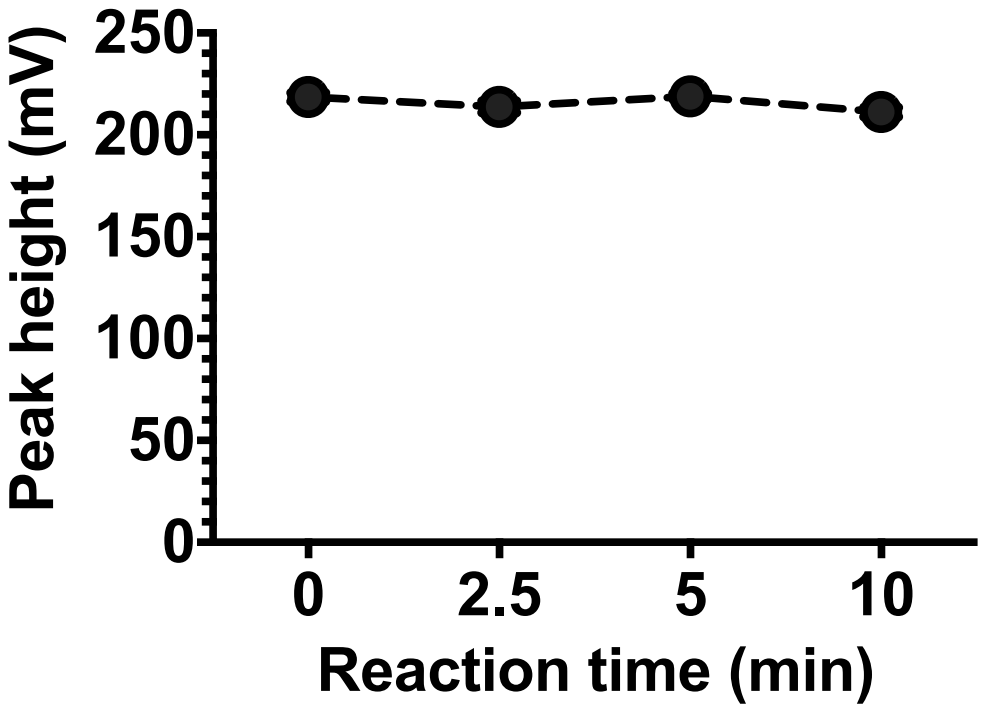

Figure 3.2 Stability of the OPA/sulfite GABA derivative up to 10 minutes

Maximum fluorescence of the derivative is immediate and stable up to 10 minutes. Data are presented as mean, sem are smaller than symbol size.

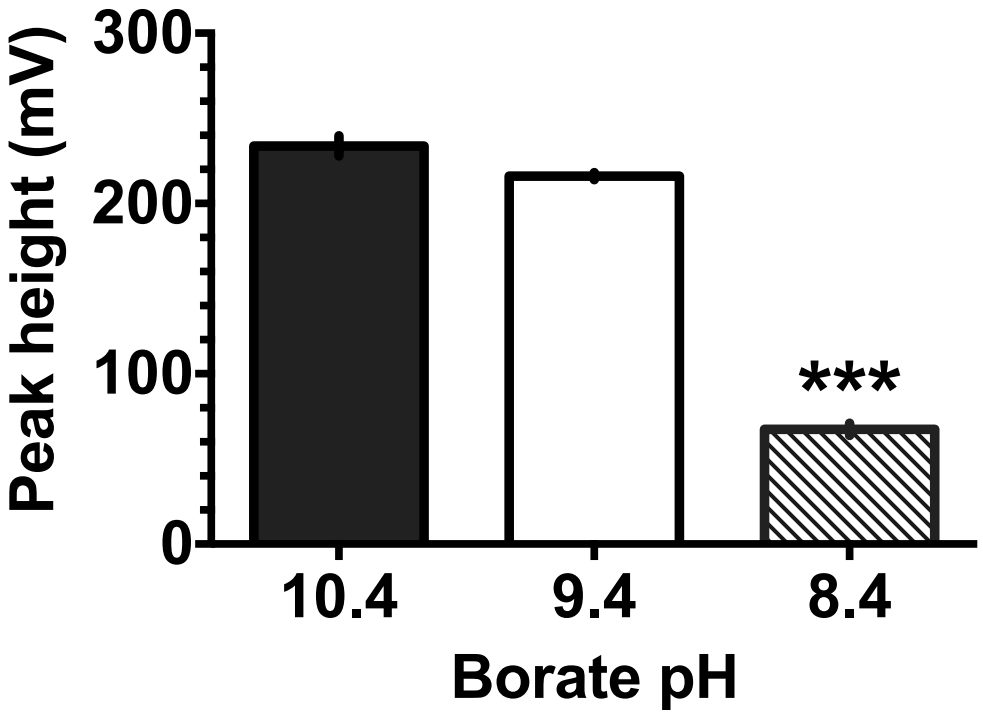

Figure 3.3 Effects of borate buffer pH in derivatization working solution on GABA signal

A pH of 8.4 significantly reduces fluorescence of the OPA/sulfite GABA derivative (*** indicates $p<0.001$). Data are presented as mean ± sem.

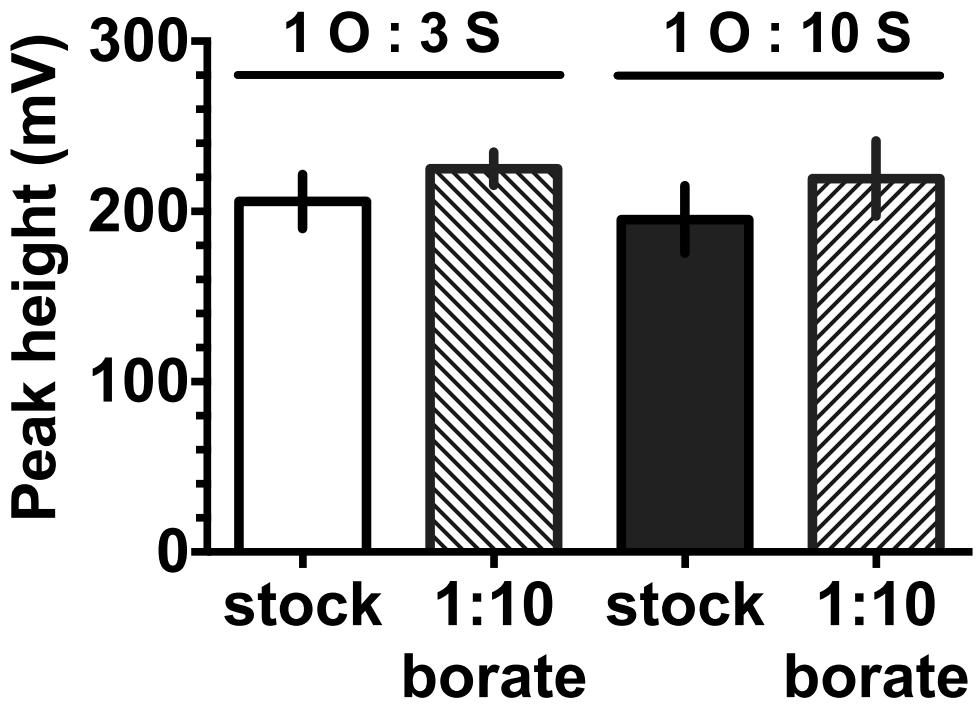

Figure 3.4 Effects of adjusting the ratio of OPA to sulfite in the derivatization solution on GABA signal

Increasing sulfite in OPA solution improved separation of GABA with no significant reduction in GABA signal. Original OPA derivatization solution consisted of a mole ratio of 1 OPA: 3 sulfite. High sulfite derivatization solution consisted of a mole ratio of 1 OPA: 10 sulfite. Signals from stock and 1:10 dilutions in borate were compared for each derivatization solution. Data are presented as mean ± sem. O=OPA; S=sulfite.

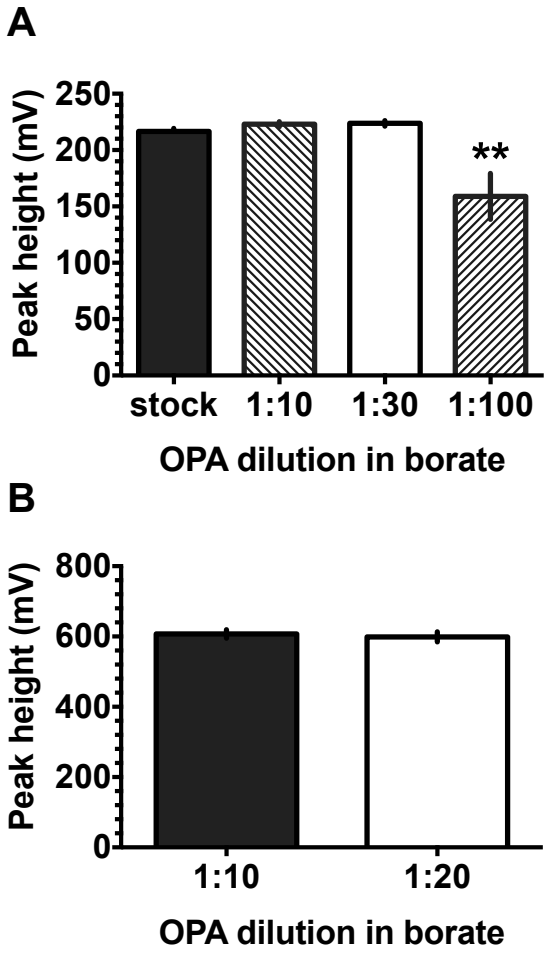

Figure 3.5 Effects of diluting the original derivatization working solution (A) or high sulfite derivatization working solution (B) in borate on GABA signal

(A) Maximal GABA signal was maintained at 1:10 or 1:30 dilution in borate while reducing additional peaks compared to stock solution. A 1:100 dilution of the derivatization working solution in borate significantly reduced the GABA signal (** indicates $p<0.01$). (B) Using a high sulfite derivatization working solution, no effects on GABA signal were observed between 1:10 and 1:20 dilution in borate. For both panels data are presented as mean ± sem.

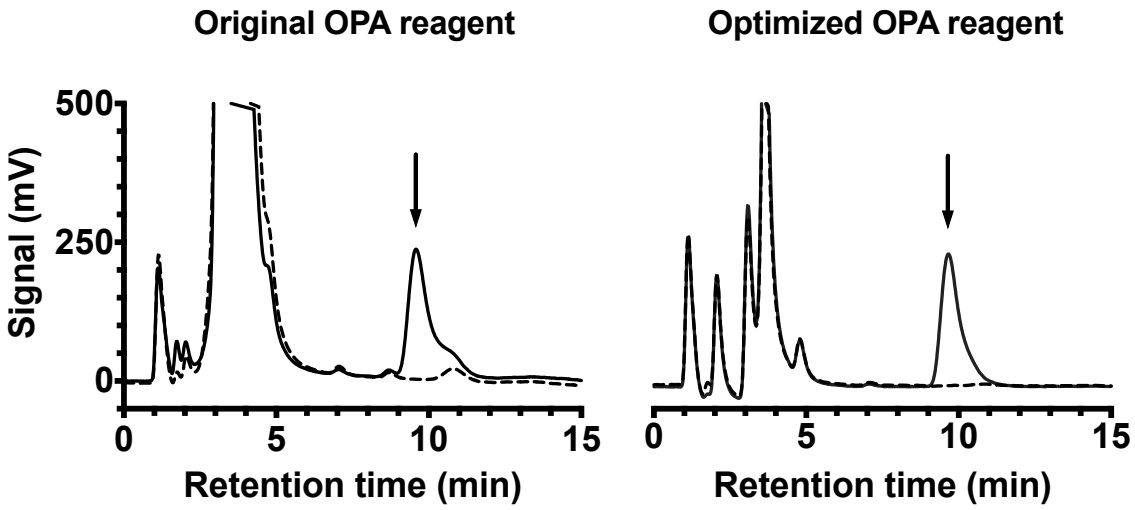

Figure 3.6 Representative chromatograms showing improved GABA peak separation by optimizing the derivatization working solution

Optimization of OPA reagent eliminated co-eluting peaks without reducing GABA signal. Dotted lines represent OPA blanks and solid lines represent GABA standard (500 nM) injections, arrows indicate GABA peak. Original OPA derivatization solution [mole ratio 1 OPA: 3 sulfite] (A) compared to high sulfite OPA derivatization solution [mole ratio 1 OPA: 10 sulfite] diluted 1:10 in borate (B).

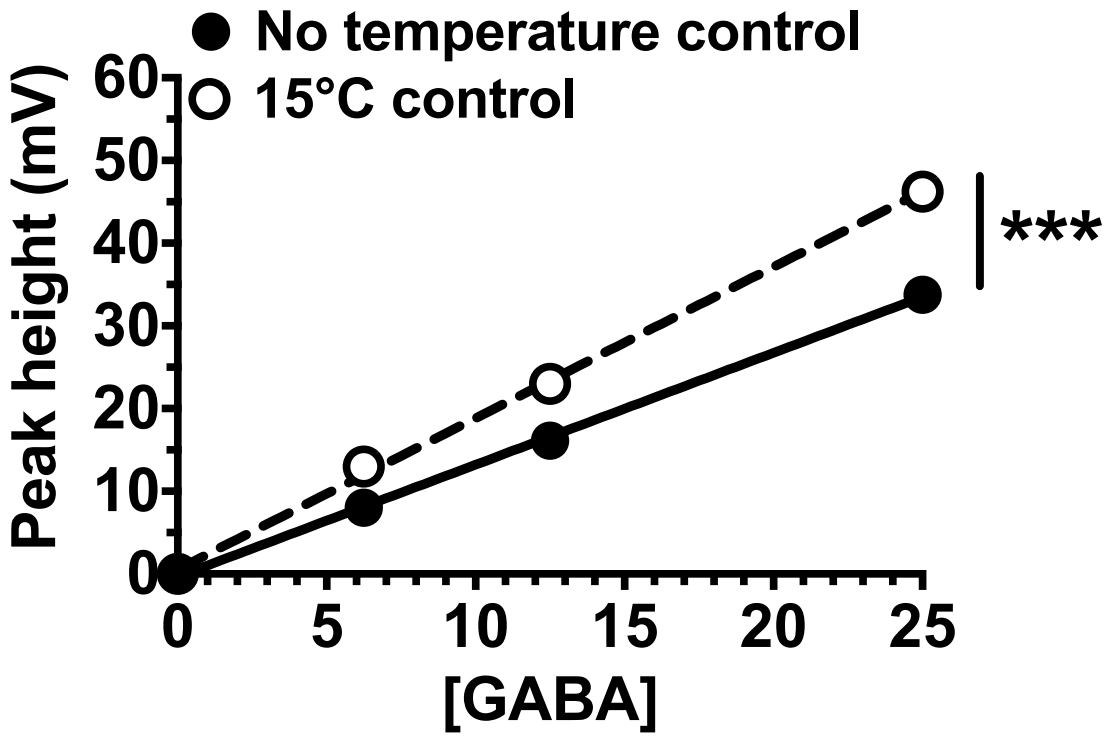

Figure 3.7 Effect of temperature on fluorescence of OPA/sulfite GABA derivative

Controlling the temperature of the detector at 15°C significantly increased GABA signal (*** indicates $p<0.001$). Data are presented as mean, sem are smaller than symbol size.

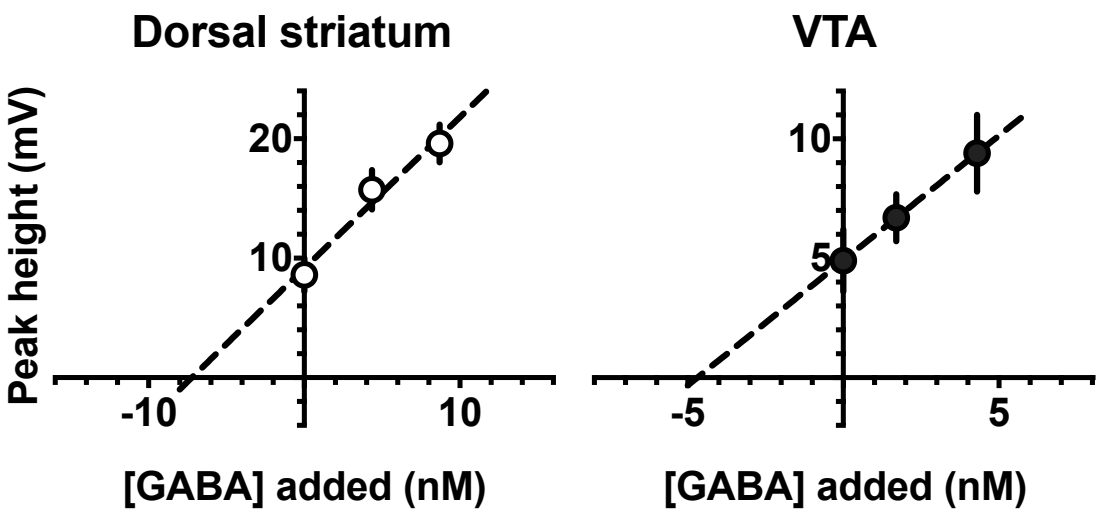

Figure 3.8 Method of standard additions using *in vivo* dialysates from dorsal striatum and ventral tegmental area of Long Evans rats

Dorsal striatum (R^2=0.97, n=4); ventral tegmental area (VTA: R^2=1.00, n=3). Values not corrected for probe recovery or dilution in borate. Data are presented as mean ± sem.

CONCLUSION

We have determined excitation and emission wavelengths that allow for a novel, sensitive fluorescence method to detect GABA using HPLC of an OPA/sulfite derivative. The derivatization solutions and procedure were optimized to achieve reliable GABA peak separation in brain microdialysis samples. We validated our method by quantifying extracellular GABA using external standards and standard additions, further confirming no interfering peaks were present in biological samples. The described method is advantageous for various neuroscience applications that require sensitive, reliable analysis of amino acids using a simplified chromatographic system.

Chapter 4: Local GABA uptake inhibition reduces *in vivo* extraction fraction in the ventral tegmental area of Long Evans rats measured by quantitative microdialysis under transient conditions

ABSTRACT

Inhibitory signaling in the ventral tegmental area (VTA) is involved in the mechanism of action for many drugs of abuse. Although drugs of abuse have been shown to alter extracellular γ-aminobutyric acid (GABA) concentration in the VTA, knowledge on how uptake mechanisms are regulated *in vivo* is limited. Quantitative (no net flux) microdialysis is commonly used to examine the extracellular concentration and clearance of monoamine neurotransmitters, however it is unclear whether this method can be used to estimate clearance for amino acid neurotransmitters such as GABA. The purpose of this study was to determine whether changes in GABA uptake are reflected by *in vivo* extraction fraction within the VTA. Using quantitative (no net flux) microdialysis adapted for transient conditions, we examined the effects of local perfusion with the GABA uptake inhibitor, nipecotic acid, in the VTA of Long Evans rats. Basal extracellular GABA concentration and *in vivo* extraction fraction were 44.4 ± 1.9 nM and 0.19 ± 0.01, respectively. At maximal effect, nipecotic acid (50 μM) significantly increased extracellular GABA concentration to 170 ± 4.1 nM and reduced *in vivo* extraction fraction to 0.11 ± 0.003. Extraction fraction returned to baseline immediately

following removal of nipecotic acid from the perfusate. Due to reduced *in vivo* extraction fraction, conventional microdialysis substantially underestimated the increase of extracellular GABA from local uptake inhibition by nipecotic acid perfusion. Together, these results suggest inhibiting GABA uptake mechanisms within the VTA alters *in vivo* extraction fraction measured using microdialysis.

INTRODUCTION

The ventral tegmental area (VTA) is a brain region involved in reward processing, aversion and the development of some drug-dependent behaviors (Fields *et al*. 2007; Lüscher and Malenka 2011; Oliva and Wanat 2016). Inhibitory signaling in the VTA is altered by many drugs of abuse and γ-aminobutyric acid (GABA) neurons have an emerging role in regulating reward and aversive learning (Bocklisch *et al*. 2013; Creed *et al*. 2014; Nugent and Kauer 2008). Despite the importance of inhibitory signaling in the VTA for drug-related behaviors, little is known how extracellular GABA concentration in the VTA is regulated.

GABA transporters (GATs) on neurons and astrocytes are critical in the regulation of GABA in the extracellular space (Scimemi 2014). Within the central nervous system, GAT-1 is the primary neuronal isoform and GAT-3 is strongly expressed in astrocytes (Borden 1996; Roth and Draguhn 2012). Inhibition of GATs reduces GABA uptake from the synaptic cleft and is a useful drug target for the treatment of epilepsy, pain and anxiety (Bröer and Gether 2012). Tiagabine, a highly selective GAT-1 inhibitor, is currently approved as an adjunctive treatment for partial epilepsy (Meldrum and Rogawski 2007). There is also clinical evidence that tiagabine may be effective in decreasing drug taking behavior, craving and withdrawal signs (González *et al*. 2007; Myrick *et al*. 2005; Sofuoglu *et al*. 2005). More recently, animal studies have shown that GATs can contribute to changes in GABAergic signaling during drug withdrawal (Bagley *et al*. 2011), and midbrain dopamine neurons also express GATs to facilitate co-release of GABA (Tritsch *et al*. 2014). Together, these results suggest the maintenance of

drug-related behaviors may involve changes in the regulation of extracellular GABA concentration in certain brain regions.

Microdialysis is one technique used to study the regulation of GABA concentration in the extracellular space. Dialysate GABA concentrations are interpreted as an index of volume transmission from the interaction between neurons and astrocyte networks rather than a fast measure of synaptic release (Del Arco *et al*. 2003; Nyitrai *et al*. 2006). However, the mechanisms of regulation of extracellular GABA in the VTA are not clear, in part due to low reported basal concentrations and specific chromatographic conditions required for accurate quantification (Fliegel *et al*. 2013; Rea *et al*. 2005). Despite these challenges, several studies have measured changes in dialysate GABA concentration within the VTA in response to acute or chronic treatment with drugs of abuse (Bankson and Yamamoto 2004; Buczynski *et al*. 2016; Klitenick *et al*. 1992; Natividad *et al*. 2012; O'Dell and Parsons 2004; Sotomayor-Zarate *et al*. 2015; Vihavainen *et al*. 2008). However, dialysate measurements may not accurately reflect changes in extracellular concentration due to the independent influence of *in vivo* extraction fraction, which can be altered by neurotransmitter clearance (Parsons *et al*. 1991). The high efficiency of GABA clearance by GATs maintains low extracellular concentrations and may interfere with accurate determination of effects on neuronal release following drug administration. Therefore, while previous work implicates changes in extracellular VTA GABA concentration in the mechanism of many drugs of abuse, it is critical to examine the influence of uptake when interpreting changes in dialysate GABA levels.

In contrast to conventional microdialysis, quantitative (no net flux) microdialysis is a technique that involves including different concentrations of the analyte sequentially within the perfusate in order to determine the point of "no net flux" across the probe membrane, indicating the actual extracellular concentration in the brain at steady state conditions (Justice 1993; Lonnroth *et al*. 1987). The *in vivo* extraction fraction, or relative recovery of the probe, is the slope of the regression line determined from the experiment and has been shown to be an estimate of neurotransmitter clearance for monoamines and acetylcholine (Parsons and Justice 1992; Smith and Justice 1994; Cosford *et al*. 1996; Vinson and Justice 1997). It is less clear whether *in vivo* extraction fraction estimates clearance for amino acid neurotransmitters that are regulated by both neurons and astrocytes. For glutamate, reduced *in vivo* extraction fraction coincided with lower protein expression of excitatory amino acid transporter 1 (EAAT1) in the VTA (Ding *et al*. 2013). However, it is currently unknown whether changes in GABA uptake are reflected by changes in microdialysis *in vivo* extraction fraction.

GABA clearance from the extracellular space is primarily mediated through uptake by transporters, therefore we hypothesized that inhibiting GATs would significantly reduce the *in vivo* extraction fraction measured using microdialysis. We used a modification of quantitative microdialysis under transient conditions (Olson and Justice 1993) to measure the direct effects of nipecotic acid, a GAT inhibitor, on extracellular GABA concentration and *in vivo* extraction fraction over time in the VTA of Long Evans rats.

METHODS

Animals

Adult, male Long Evans rats (n=32; Harlan, Indianapolis, IN) were maintained in a humidity and temperature controlled room with a 12-hour light/dark cycle (lights on at 0700). Animals were pair housed and handled daily for at least one week to acclimate to facility prior to surgery. Food and water were available *ad libitum*. All animal procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals (8th Ed., 2011) and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Materials

Nipecotic acid, GABA, *o*-phthalaldehyde (OPA), sodium dihydrogen phosphate dihydrate, sodium sulfite and sodium tetraborate decahydrate were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO). Methanol from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ) and absolute ethanol from AAPER (AAPER Alcohol and Chemical Co., Shelbyville, KY) were used. All solutions were made with deionized water from a Milli-Q system (Millipore, Billerica, MA) and filtered using 0.2 μm nylon filters (Pall Corp., Ann Arbor, MI). Gentamicin (APP Pharmaceuticals, Schaumburg, IL) and carprofen (Pfizer, New York, NY) were used in surgery. Nipecotic acid and GABA were dissolved in artificial cerebrospinal fluid (ACSF) for microdialysis experiments.

Microdialysis surgery

Rats were anesthetized with isoflurane (5% induction, 2.5% maintenance) and guide cannulae (21 gauge, Plastics One, Roanoke, VA) were stereotaxically implanted above the VTA (mm relative to bregma and skull surface: anteroposterior -5.8, mediolateral +2.1, ventral -4.6 at 10° angle toward midline) according to atlas of Paxinos and Watson (2007). Probes extended 4.0 mm below the end of cannula when implanted for experiments. An obturator was placed in the guide cannula until probe implantation. Carprofen (5 mg/kg, subcutaneous) was given during surgery to minimize post-operative pain and discomfort. After surgery, rats were housed individually and underwent a recovery period of at least 5 days prior to microdialysis experiments.

Microdialysis procedures

Microdialysis probes (1 mm active area, 13 kDa molecular weight cutoff) were constructed similar to Pettit and Justice (1991b), and experimental conditions were described previously (Doyon *et al*. 2003; Valenta *et al*. 2013). Rats (328-495 g on experiment day) were lightly anesthetized and probes inserted into cannula 12-18 hours prior to experiments. Probes were perfused with ACSF (149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.4 mM D-glucose) at 0.2 µl/min overnight and then the flow rate was increased to 1.0 µl/min at least two hours prior to the start of microdialysis experiments. Following this equilibration period, samples were collected every 20 minutes and all experiments occurred between 0700 – 1700 hours, during the light phase of the cycle.

Experiment 1

We first conducted an experiment to determine the concentration of the GABA uptake inhibitor nipecotic acid to perfuse through the probe for the quantitative microdialysis experiment. Following a baseline period, the probe perfusate (ACSF) was manually changed to a syringe containing nipecotic acid in ACSF (30, 100, 300 μM) in sequential order. Each nipecotic acid concentration was perfused for 60 minutes. After the final nipecotic acid concentration, the perfusate was changed back to ACSF for three additional samples. In control animals, the syringe was changed to a different syringe containing only ACSF with no inhibitor at each time point. The range of concentrations tested for nipecotic acid was chosen based on previous reports (Rea *et al*. 2005; Vihavainen *et al*. 2008).

Experiment 2

A modification of the quantitative microdialysis (no net flux) adapted for transient conditions was used to determine the effects of local uptake inhibition on extracellular GABA concentration and *in vivo* extraction fraction (Lonnroth *et al*. 1987; Olson and Justice 1993). Probes were perfused with ACSF overnight as described above. The morning of the experiment, the perfusate was changed to ACSF including one of four GABA concentrations (0, 15, 45, 90 nM) and the flow rate increased to 1.0 μl/min for at least two hours. These GABA concentrations were chosen based on preliminary no net flux experiments (data not shown). After four baseline samples, the perfusate was manually changed to ACSF containing the same GABA concentration and nipecotic acid

(50 μM) for five samples, then returned to the baseline ACSF solution for four samples. Dialysis samples were immediately frozen on dry ice and stored at -80°C until analysis.

GABA analysis

All samples underwent precolumn derivatization prior to analysis. The derivatization solution was based on previous reported methods (Rowley *et al*. 1995; Smith and Sharp 1994). The working solution was made by dissolving 11 mg OPA in 250 μl absolute ethanol, 250 μl sodium sulfite (1 M) and 4.5 ml sodium tetraborate decahydrate (0.1 M, adjusted to pH 10.4 with 5 M NaOH; borate). The working solution was stored in a covered vial at 4°C. The derivatization procedure consisted of combining 9 μl standard or sample with 2 μl borate then adding 0.5 μl OPA working solution, which was manually mixed and incubated in darkness at room temperature for 10 minutes. Stock GABA standards were prepared in deionized water and stored at 4°C for up to one month. Serial dilutions were prepared fresh daily.

The GABA concentration in samples was determined using high performance liquid chromatography with electrochemical detection. All samples were run with external standards. For the quantitative microdialysis experiment, GABA concentration in the ACSF perfusate for each rat was validated using external standards. Samples were run using a 9725i manual injector (Rheodyne, Cotati, CA), Luna C18(2) column (150 x 2.0 mm, 3 μm; Phenomenex, Torrance, CA), Decade controller (column temperature 40-45°C; Antec Leyden, Zoeterwoude, Netherlands) and 2 mm glassy carbon working electrode (SenCell or VT-03 with salt bridge Ag/AgCl reference; Antec) at +700 mV

potential. Aqueous mobile phase consisted of 0.1 M sodium dihydrogren phosphate dihydrate and 0.5 mM EDTA adjusted to pH 4.50 with 1 M phosphoric acid. Methanol was added (27% v/v) to mobile phase and sparged with helium prior to being pumped at a flow rate of 0.14-0.25 ml/min through the system. Following precolumn derivatization, 5-10 µl of the reaction mixture was manually injected into the system. Chromeleon 6.8 Chromatography Data System Software (Thermo Fisher Scientific, Waltham, MA) was used for chromatogram acquisition and peak integration. Chromatographic peaks were required to have a signal to background noise ratio of at least 2:1 in Experiment 1 and 3:1 in Experiment 2 to be included in the analyses.

Histological analysis

After microdialysis experiments, animals were administered a lethal dose of sodium pentobarbital (150 mg/kg, intraperitoneal) under isoflurane anesthesia. Brains were extracted and stored in 10% formalin. Coronal sections (120 µm) were stained with cresyl violet for probe verification (Paxinos and Watson 2007). Site of probe implantation was determined prior to data analysis. The probe active area was required to be located in the VTA and adjacent midbrain for animals to be included in the study.

Statistical analysis

Experiment 1

Microdialysis data were expressed as percent of baseline from the average of the first three samples and analyzed using repeated measures ANOVA with perfusate treatment as a between-subjects factor and time as a repeated measure.

Experiment 2

Quantitative microdialysis data were analyzed according to procedures described in Yim and Gonzales (2000). There were four subgroups (each subgroup, n=5-7) based on the concentration of GABA (0, 15, 45, 90 nM) in the perfusate during the experiment. Data from subgroups were used to create a linear regression for each time point by plotting $[GABA]_{in}$-$[GABA]_{out}$, the difference between the concentration perfused through the probe ($[GABA]_{in}$) and the concentration in the sample ($[GABA]_{out}$) compared with $[GABA]_{in}$ (Olson and Justice 1993). Linear regression was performed for each time point to calculate the extracellular GABA concentration (x-intercept) and *in vivo* extraction fraction (slope). The asymptotic standard errors for regression parameters were determined from the linear regression. The method of extra sum of squares was used to test if there were differences between regression lines (Kenakin 1997). If significant differences between regression lines were observed, additional F tests were carried out to determine whether changes in the x-intercept, slope or both parameters contributed to the difference.

For all analyses, Bonferroni's post hoc comparisons were used as appropriate. Values are reported as mean ± sem and results with $p<0.05$ were assigned significance. Data analysis was performed using GraphPad Prism (version 6; La Jolla, CA) or SPSS software (version 22; IBM, Armonk, NY).

RESULTS

Histological analysis

The probe locations were verified as shown in Fig. 4.1, and ranged from approximately 5.28 to 6.36 mm posterior from bregma according to Paxinos and Watson (2007). In the quantitative microdialysis experiment (Fig. 4.1B), 87% of probe placements were located in the posterior VTA (defined as -5.6 to -6.3 mm from bregma) (Sanchez-Catalan *et al*. 2014).

Experiment 1: Dose dependent effects of local uptake inhibition on GABA release using conventional microdialysis

In order to determine the concentration of nipecotic acid to use for the quantitative microdialysis experiment, we first tested the effects of three different concentrations (30, 100, 300 μM) of nipecotic acid, a GABA uptake inhibitor, on VTA GABA dialysate concentration using local application through the microdialysis probe. A control group of animals received only ACSF throughout the experiment but manual syringe changes occurred at same times as the group that received increasing

concentrations of nipecotic acid in the perfusate. The overall ANOVA revealed a significant interaction between treatment group and time ($F_{14,84}$=40.7, p<0.0001). There was a significant difference over time in VTA GABA concentration as a percent of baseline in the group that received local nipecotic acid application (Fig. 4.2; $F_{14,42}$=47.15, p<0.0001; n=4). Post hoc analyses revealed GABA concentration significantly increased due to nipecotic acid treatment between 140 – 240 minutes compared to controls (all p<0.001). Additionally, the GABA concentration in the first ACSF sample following the final nipecotic acid concentration (300 μM) was significantly elevated compared to controls (260 min, p<0.05). In contrast, GABA concentration did not significantly change over time in control animals as a result of manual ACSF syringe changes ($F_{14,42}$=0.69, ns; n=4).

Experiment 2: Effect of local uptake inhibition on extracellular GABA and *in vivo* extraction fraction using quantitative microdialysis under transient conditions

The results of Experiment 1 indicated a concentration higher than 30 μM nipecotic acid was necessary to significantly increase VTA GABA over baseline. Therefore, we tested the effect of 50 μM nipecotic acid delivered through the probe on extracellular GABA and *in vivo* extraction fraction in the VTA using quantitative microdialysis under transient conditions (Olson and Justice 1993). A separate group of animals was used for each GABA concentration in the ACSF perfusate (GABA (nM): 0, n=6; 15, n=5; 45, n=7; 90, n=6).

The extracellular concentration of GABA and *in vivo* extraction fraction were calculated using linear regression, as illustrated by selected time points in Fig. 4.3. A positive value on the y-axis indicates net diffusion of GABA into the brain and a negative number indicates net GABA diffusion from the brain into the perfusate (Parsons and Justice 1992). The time course of extracellular GABA concentrations and *in vivo* extraction fraction are presented in Fig. 4.4. Analysis of the regression parameters between the four baseline time points revealed no significant differences (Fig. 4.3A; $F_{6,8}$=0.88, ns). The average extracellular GABA concentration in the VTA and *in vivo* extraction fraction during the baseline were 44.4 ± 1.9 nM and 0.19 ± 0.01, respectively.

During the nipecotic acid perfusion, there was a significant difference observed in the regression line at each time point 100 – 180 minutes compared to baseline regressions (100 min, $F_{8,10}$=17.9; 120 min, $F_{8,10}$=25.6; 140 min, $F_{8,10}$=106.3; 160 min, $F_{8,10}$=89.5; 180 min, $F_{8,10}$=173.7; all $p<0.001$). Local nipecotic acid application significantly increased the extracellular GABA concentration (x-intercept) over baseline samples (Fig. 4.4; 100 min, $F_{4,10}$=35.7; 120 min, $F_{4,10}$=46.5; 140 min, $F_{4,10}$=174.2; 160 min, $F_{4,10}$=141.6; 180 min, $F_{4,10}$=257.6; all $p<0.001$). In addition to the significant increase in extracellular GABA, nipecotic acid also significantly reduced the *in vivo* extraction fraction (slope) in most samples during the perfusion (Fig. 4.4; 100 min, $F_{4,10}$=3.76; 140 min, $F_{4,10}$=5.34; 160 min, $F_{4,10}$=5.90; 180 min, $F_{4,10}$=13.28; 100, 140 – 160 min $p<0.05$, 180 min $p<0.01$). Both effects were maximal following 100 minutes of nipecotic acid perfusion. At this time point (180 min), extracellular GABA concentration increased to 170 ± 4.1 nM and *in vivo* extraction fraction had fallen by approximately 40% (0.11 ± 0.003).

After 100 minutes of nipecotic acid perfusion, the syringes were changed back to the original ACSF solution. There was a significant difference between the baseline regressions and the regression at the first time point following the nipecotic acid perfusion (200 min, $F_{8,10}$=9.73, p<0.001). At this time point (200 min), the extracellular GABA concentration (x-intercept) was still significantly elevated ($F_{4,10}$=19.1; p<0.001) but the *in vivo* recovery (slope) had returned to baseline ($F_{4,10}$=0.72; ns). There were no significant differences between the four baseline regressions and the last 60 minutes of the experiment (Fig. 4.4; 220 min, $F_{8,20}$=0.91, ns; 240 min, $F_{8,20}$=0.84, ns; 260 min, $F_{8,20}$=0.59; ns). The average extracellular GABA concentration and *in vivo* extraction fraction did not differ between baseline periods before and after the nipecotic acid perfusion (x-intercept: t(5)=1.21, ns; slope: t(5)=0.39, ns).

Figure 4.5 compares the time course of nipecotic acid perfusion on $[GABA]_{out}$ from the group that received 0 nM GABA in the perfusate to the extracellular GABA concentration determined from quantitative microdialysis as percent of baseline from the average of the four baseline time points. At the maximal effect, $[GABA]_{out}$ increased to 230% of baseline while the extracellular concentration increased to almost 400% of baseline. Therefore, our results indicate conventional microdialysis underestimates the increase in extracellular GABA concentration measured following uptake inhibition due to the decrease in extraction fraction.

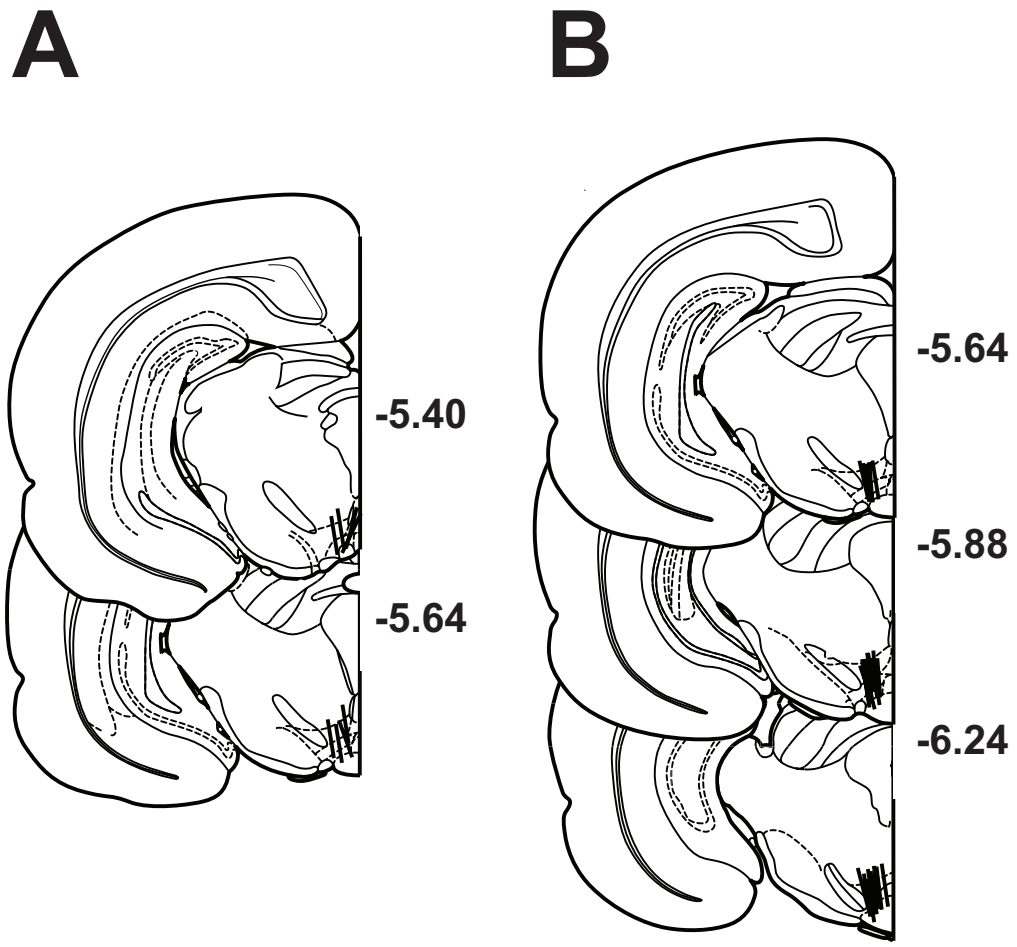

Figure 4.1 Microdialysis probe placements within the ventral tegmental area

Coronal sections showing representative probe placements for Experiment 1 (A) and Experiment 2 (B). Lines represent 1 mm active dialysis area. Numbers to the right indicate distance relative to bregma (mm). Histology figure adapted from Paxinos and Watson (2007).

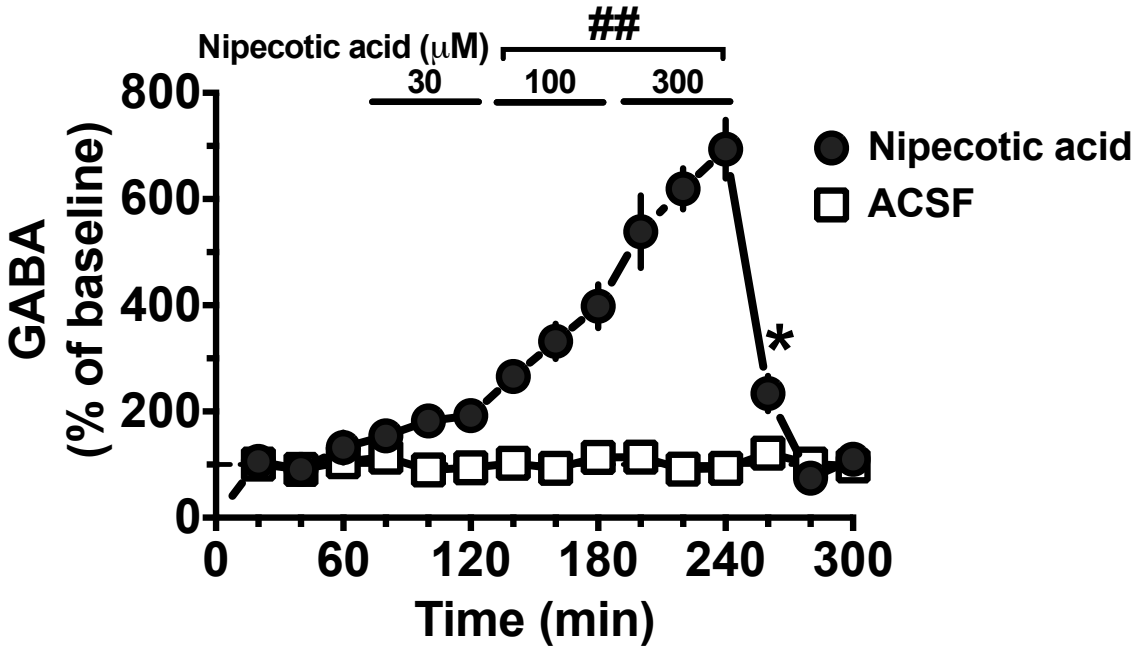

Figure 4.2 Effect of local uptake inhibition by nipecotic acid in microdialysis perfusate on GABA concentration in the VTA

Local application of 100 and 300 μM nipecotic acid significantly increased GABA concentration compared to control rats (* indicates $p<0.05$, ## indicates $p<0.001$). Each concentration of nipecotic acid (30, 100, 300 μM) was administered in sequential order for 60 minutes (n=4). In control rats, manual syringe changes occurred at the same times as the nipecotic acid group but syringes only contained artificial cerebrospinal fluid (ACSF; n=4). Data are presented as mean ± sem, sem smaller than symbol size for some data points.

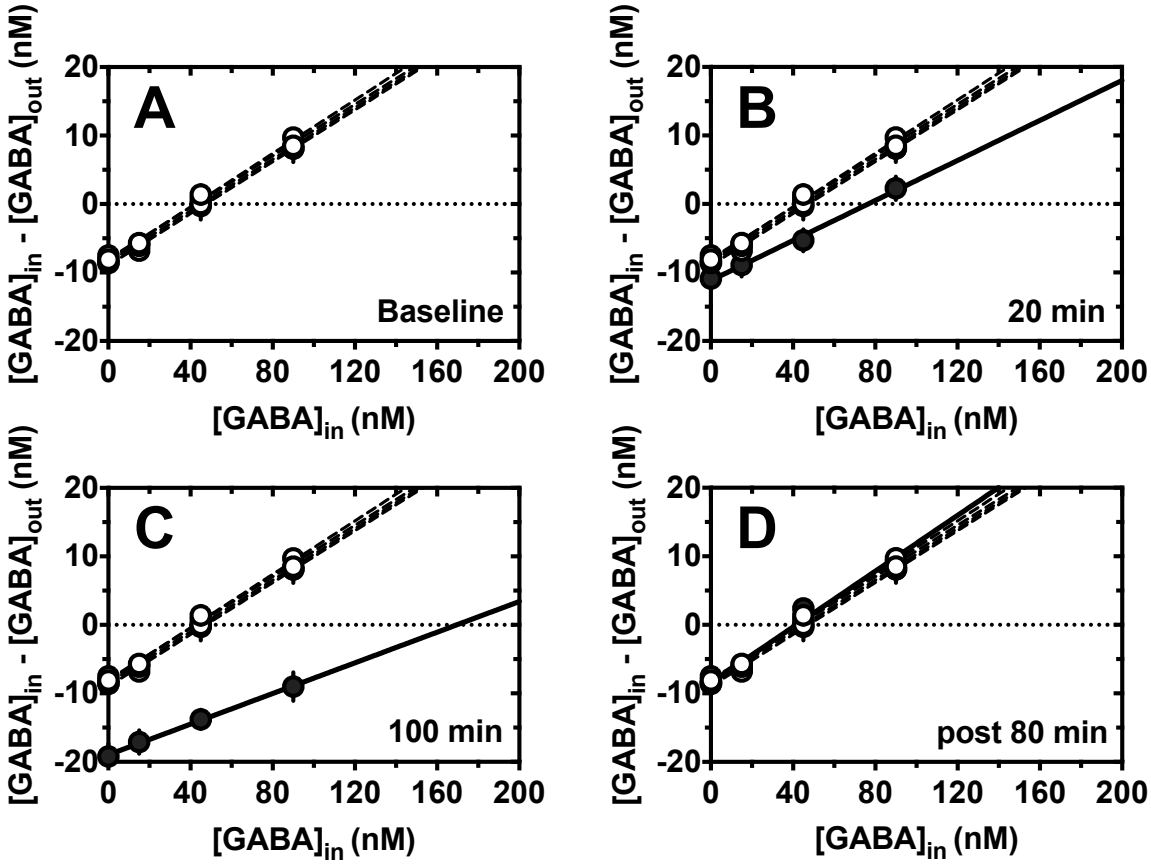

Figure 4.3 Regression lines showing extracellular concentration and extraction fraction for samples collected during baseline, during nipecotic acid perfusion and return to baseline

The effect of four GABA concentrations (0, 15, 45, 90 nM) in the perfusate ($[GABA]_{in}$) was tested in four separate groups. The difference between $[GABA]_{in}$ and concentration in sample ($[GABA]_{out}$) was plotted against $[GABA]_{in}$ for the four groups at each time interval. Each panel illustrates the data and regression lines for four basal samples (open circles, dashed lines) with one regression obtained during or after local nipecotic acid (50 μM) perfusion (n=24 for combined data). (A) Comparison of the four regressions during baseline period. (B) The regression line for the first sample following 20 minutes perfusion with nipecotic acid compared to baseline. (C) The regression line for the last

sample after 100 minutes perfusion with nipecotic acid compared to baseline. (D) The regression line for the sample 80 minutes following removal of nipecotic acid in perfusate compared to baseline. The r^2 values for the basal regressions are ≥ .99. The r^2 values for the time points during and after the nipecotic acid perfusion are .91 – 1.00. Data are presented as mean ± sem for each GABA concentration.

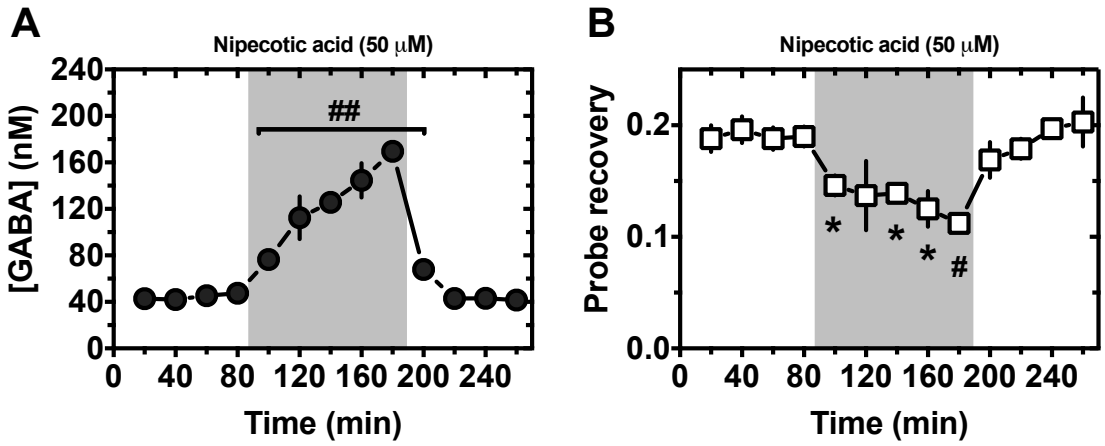

Figure 4.4 Time course of extracellular GABA and *in vivo* extraction fraction during local nipecotic acid perfusion in the ventral tegmental area

Following four baseline samples, the artificial cerebrospinal fluid (ACSF) perfusate was changed to include nipecotic acid (50 μM; shaded area) for 100 minutes and then back to ACSF for the last four samples. The slopes and x-intercepts of the linear regression lines over the course of the experiment are presented. Error bars represent the asymptotic standard errors at each time point obtained from regression statistics. Nipecotic acid perfusion significantly increased the extracellular concentration of GABA (A) compared to baseline samples (## indicates $p<0.001$ for all samples during 100 – 200 minutes). Additionally, nipecotic acid significantly reduced *in vivo* extraction fraction (B) compared to baseline samples (* indicates $p<0.05$, # $p<0.01$). The r^2 values for the individual regressions were .91 – 1.00.

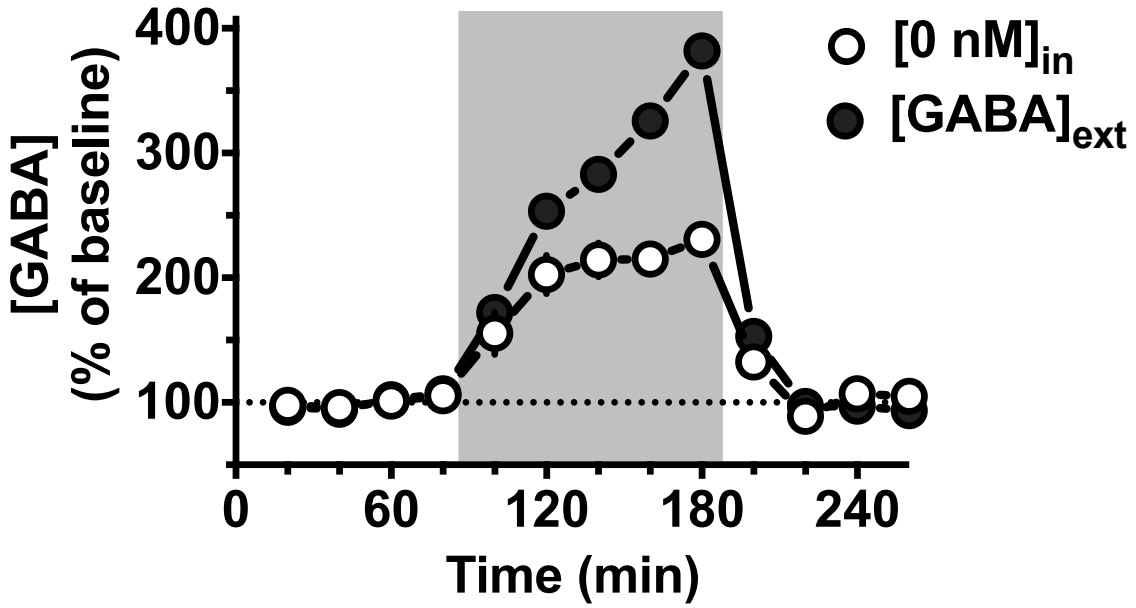

Figure 4.5 Time course of the percent baseline change of $[GABA]_{out}$ for the group receiving 0 nM GABA and extracellular GABA calculated from quantitative microdialysis during local nipecotic acid perfusion in the ventral tegmental area

The percent baseline of x-intercept from the linear regression lines over the course of the experiment are presented for extracellular GABA ($[GABA]_{ext}$; filled circles; n=24). The percent baseline of group receiving 0 nM GABA ($[0\ nM]_{in}$; open circles; n=6) in the perfusate are presented as mean ± sem. Shaded area indicates local nipecotic acid perfusion (50 μM).

DISCUSSION

The present results provide the first neurochemical evidence that inhibiting GATs significantly reduces *in vivo* extraction fraction using quantitative microdialysis. Delivery of nipecotic acid through the microdialysis probe significantly increased extracellular VTA GABA while reducing *in vivo* extraction fraction. The decrease in extraction fraction returned to baseline within the first sample following removal of the uptake inhibitor. As a result of reduced *in vivo* extraction fraction, conventional microdialysis substantially underestimated the increase in extracellular GABA concentration observed following local GAT inhibition. Consistent with measurements for monoamines, these results suggest *in vivo* extraction fraction reflects changes in GABA clearance that may influence the interpretation of the effects of drugs on dialysate GABA concentration within the VTA.

The principle finding of this study is that *in vivo* extraction fraction is sensitive to changes in GABA uptake in the VTA using quantitative microdialysis. Quantitative microdialysis has been applied extensively to determine actual extracellular concentrations of neurotransmitters during acute or following chronic drug administration. Previous studies have shown that extraction fraction, or *in vivo* recovery of the microdialysis probe, represents an indirect measure of clearance for monoamines and acetylcholine (Cosford *et al*. 1996; Parsons *et al*. 1991; Parsons and Justice 1992; Smith and Justice 1994; Vinson and Justice 1997). Changes in dopamine uptake measured by *in vivo* extraction fraction have further been confirmed using voltammetry (Chen 2005). To our knowledge, the current results are the first to extend these findings

that *in vivo* extraction fraction measured using microdialysis may also be an estimate of GABA clearance.

While there are multiple mechanisms present for release and uptake of amino acid neurotransmitters such as glutamate and GABA, in support of our findings a previous study showed reducing glutamate uptake by local application of an inhibitor through the microdialysis probe significantly reduced *in vivo* extraction fraction in the hippocampus (Chefer *et al*. 2011). However, there are mixed results on whether *in vivo* extraction fraction in quantitative microdialysis studies corresponds to changes in either glutamate transporter function or protein expression (Ding *et al*. 2013; Melendez *et al*. 2005; Pati *et al*. 2016; Shen *et al*. 2014).

Using quantitative microdialysis under transient conditions, we were able to show *in vivo* extraction fraction was significantly reduced in the first sample following GAT inhibition and returned to baseline levels following removal of the drug. These results suggest that small changes in GABA uptake can be detected easily using this technique, which is advantageous when investigating the mechanism of action of drugs as extraction fraction can change over time (Cosford *et al*. 1994). Additionally, local perfusion through the probe in the absence of other drugs or chronic treatments (e.g. cocaine, ethanol) eliminates the potential confound of other release mechanisms potentially contributing to the observed change in extraction fraction. However, we did not compare our results to extraction fraction of the probe *in vitro* or assess *in vivo* GAT function in this study so future studies are necessary to directly measure whether *in vivo* extraction fraction can be used as a measure of GABA clearance.

The activity of GATs to regulate synaptic transmission and prevent neuronal spillover is critical to maintain extracellular GABA levels. Consistent with previous reports, nipecotic acid perfusion caused a dose-dependent increase in extracellular GABA concentration measured with conventional microdialysis (DeGroote and Linthorst 2007; Rea *et al*. 2005; Vihavainen *et al*. 2008; Westerink and de Vries 1989). Our results from the quantitative microdialysis experiment further indicate reduced *in vivo* extraction fraction following GAT inhibition leads to an underestimation in the increase in extracellular GABA measured by conventional microdialysis. It is possible a portion of the extracellular GABA measured is due to mechanisms other than inhibiting uptake as nipecotic acid has previously been reported able to release GABA stores through heteroexchange at higher concentrations (Johnston *et al*. 1976; Solís and Nicoll 1992; Szerb 1982). However, only a fraction of the concentration of nipecotic acid used in our study crosses the probe membrane therefore possible effects on heteroexchange are likely minimal. Nipecotic acid potently inhibits both GAT-1 and GAT-3 on neurons and astrocytes (Krogsgaard-Larsen *et al*. 1987; Madsen *et al*. 2010). Both GAT-1 and GAT-3 isoforms are expressed in the VTA, although little is known on the distribution and heterogeneity in function that may contribute to the regulation of extracellular GABA in this region (Borden 1996; Durkin *et al*. 1995). The contribution of GAT-1 and GAT-3 to maintain extracellular GABA concentration differs between brain regions (Dalby 2000; Kersanté *et al*. 2013), therefore additional studies are needed to determine the role of individual GAT isoforms in maintaining extracellular GABA levels in the VTA.

The range of reported basal dialysate GABA concentrations in the VTA measured using conventional microdialysis varies significantly (Fliegel *et al*. 2013). Using quantitative microdialysis, the extracellular VTA GABA concentration in our study was approximately 45 nM. These results confirm extracellular GABA is maintained in the VTA at low concentrations similar to other brain regions (Wlodarczyk *et al*. 2013; Xi *et al*. 2003). The majority of the probes in the quantitative microdialysis experiment were located in the posterior VTA. The posterior VTA was targeted due to its role in the rewarding effects of many drugs of abuse (Sanchez-Catalan *et al*. 2014). However, due to the heterogeneity in GABAergic inputs to and local interneurons within the VTA (Oliva and Wanat 2016), it is possible the extracellular GABA concentration may differ for other subregions. For example, a recent study used low-flow push-pull perfusion to measure the extracellular concentration of multiple neurotransmitters with higher spatial resolution in the brain (Slaney *et al*. 2013). These authors determined the GABA concentration in the VTA to be 160 ± 50 nM, and noted significant differences between their results and extracellular concentrations previously reported for amino acid neurotransmitters in other brain regions. It is possible lack of *in vivo* calibration for the push-pull method, probe placement or greater tissue damage from the microdialysis probe could contribute to the difference in extracellular concentration compared to our current results.

Importantly, basal extracellular GABA concentrations are derived from both neuronal and non-neuronal sources that do not meet some classical criteria for exocytotic release (Del Arco *et al*. 2003; Timmerman and Westerink 1997; van der Zeyden *et al*.

2008). In addition to the current results using nipecotic acid perfusion, our preliminary experiments demonstrated a potassium-evoked increase and a reduction following baclofen perfusion on VTA GABA concentrations (data not shown). We did not test the sensitivity of extracellular GABA to calcium omission or tetrodotoxin in the current experiment, therefore we are unable to determine the portion of extracellular concentration derived from neuronal sources. However, a previous study found dialysate GABA concentration stimulated by nipecotic acid perfusion was reduced by approximately 50% following co-administration with tetrodotoxin (Vihavainen *et al*. 2008). In contrast to glutamate transporters, GATs are primarily located on neurons (Zhou and Danbolt 2013). These data suggest inhibiting GATs prior to drug administration or manipulation may provide a better index for changes in neuronal GABA release by measuring synaptic spillover. Based on our current results, the direct effects from GAT inhibitors on *in vivo* extraction fraction underestimates changes in dialysate concentration that may be observed following experimental manipulation using this technique. Additionally, there may also be effects on GABA clearance mechanisms from drug administration itself within the experiment that could influence the *in vivo* extraction fraction and interpretation of microdialysis results.

Taken together, the present results are the first to demonstrate inhibiting GABA uptake significantly reduces *in vivo* extraction fraction as measured by quantitative microdialysis in the VTA. Our results indicate that *in vivo* extraction fraction may be a critical factor when interpreting microdialysis data examining the mechanism of action of drugs on extracellular GABA concentration. Extracellular GABA concentration can alter

membrane expression and mobility of GATs influencing the strength of GABAergic signaling in the brain (Scimemi 2014). Using quantitative microdialysis, examining *in vivo* extraction fraction changes over time may be a valuable tool to investigate how GABA uptake mechanisms are regulated and better define the role of extracellular GABA in the VTA in mediating the effects of drugs of abuse or expression of drug-dependent behaviors.

Chapter 5: Concluding remarks and future studies

NALTREXONE EFFICACY IN AN ADOLESCENT RAT MODEL

The first experiment in this dissertation examined the efficacy of systemic naltrexone to reduce operant ethanol self-administration behaviors in an adolescent animal model. Our results show that naltrexone significantly reduced operant ethanol self-administration and "relapse" seeking of ethanol in rats that began drinking in adolescence to a similar degree as rats that began drinking in adulthood. Our findings extend current work that has shown naltrexone decreases operant ethanol self-administration in adult animal models (Ciccocioppo *et al*. 2003; Gonzales and Weiss 1998; Hay *et al*. 2013; Henderson-Redmond and Czachowski 2014). These results also provide preclinical evidence to support the initial positive results from clinical use of naltrexone in reducing alcohol drinking in adolescent and young adults (Miranda *et al*. 2014; O'Malley *et al*. 2015).

We hypothesize that opioid receptor antagonism is the mechanism by which naltrexone reduced ethanol self-administration in rats that began operant ethanol self-administration in adolescence. Limited evidence suggests there are no differences in MOR expression in the nucleus accumbens across adolescence in Long Evans rats, although the VTA was not examined (Ellgren *et al*. 2008). Interestingly, beta-endorphin levels in the VTA were reduced in adolescent rats with home cage access to ethanol

(Palm and Nylander 2014). Therefore, future studies are still required to determine MOR expression and function within the VTA during adolescence.

Opioid receptor antagonism attenuates ethanol-stimulated dopamine release in the nucleus accumbens during ethanol self-administration (Gonzales and Weiss 1998; Middaugh *et al*. 2003). During development, dopamine neuron firing rate in the VTA peaks in late adolescence (McCutcheon and Marinelli 2009; McCutcheon *et al*. 2012). While some microdialysis studies have suggested basal extracellular dopamine concentration in the nucleus accumbens shows a similar pattern (Badanich *et al*. 2006; Philpot *et al*. 2009), other studies have reported no differences (Frantz *et al*. 2007; Natividad *et al*. 2010; Natividad *et al*. 2012). However, there are currently no investigations monitoring dopamine concentration during operant ethanol self-administration in rats that began drinking in adolescence.

The conventional mechanism by which naltrexone is thought to attenuate ethanol-stimulated dopamine release is due to antagonism of MORs on GABAergic neurons within the VTA, or possibly RMTg (Johnson and North 1992; Lecca *et al*. 2011; Lecca *et al*. 2012). However, dopamine neurons in the VTA possess a different composition of opioid receptors dependent on projection target and not all GABAergic interneurons are inhibited by MOR agonists (Ford *et al*. 2006; Margolis *et al*. 2008; Margolis *et al*. 2012). Previous studies have shown effects on postsynaptic kappa opioid receptors can be separated by projection target for VTA dopamine neurons (Ford *et al*. 2006; Margolis *et al*. 2006a; Margolis *et al*. 2008). Therefore, the effects of MORs on dopamine and GABA neurons may also sort by projection target. To our knowledge, there are no studies

investigating whether the effects of nonselective opioid antagonism are also heterogeneous within the VTA. However, it is likely that systemic naltrexone administration produces cell-specific effects in the VTA that would then lead to differential alteration of neuronal circuits that depend on the projection target from the VTA. Future work is still needed to determine which neuronal circuits contribute to the reduction in ethanol consumption observed following naltrexone administration.

One potential mechanism underlying effects of opioid receptor blockade on ethanol-stimulated dopamine release following chronic ethanol consumption is an increase in extracellular GABA concentration in the VTA. MOR knockout mice display significantly higher dialysate GABA concentration, suggesting opioid tone is present within the VTA under basal conditions (Chefer *et al*. 2009a). Additionally, opioid peptide levels in the VTA were reduced following chronic ethanol consumption (Palm and Nylander 2014). Recently, nonoperant ethanol self-administration in adolescence was shown to increase GABAergic transmission onto VTA dopamine neurons in adulthood (Schindler *et al*. 2016). Our current results show naltrexone significantly reduced ethanol intake in both age groups, however rats that began operant self-administration in adolescence displayed less "relapse" to ethanol seeking behavior in adulthood. Therefore, future studies should examine whether age-related initiation of ethanol consumption contributes to differences in neurochemical responses to ethanol or naltrexone during operant self-administration. These experiments may provide further insight into developing future treatments for alcohol dependence resulting from early onset of problematic drinking.

It is important to emphasize that most animal experiments including the current results show naltrexone reduces ethanol consumption following acute administration. However, naltrexone is most often used clinically to reduce alcohol drinking as a chronic treatment. While no differences were found in extracellular naltrexone concentration between acute or chronic treatment over 10 days (Burattini *et al*. 2008), chronic naltrexone treatment has been shown to upregulate MOR receptors throughout the brain, including in the VTA (Lesscher *et al*. 2003). Indeed, some studies have shown that repeated or chronic administration of naltrexone can result in tolerance in the ability to reduce ethanol consumption in animal models (Boyle *et al*. 1998; Myers *et al*. 1986; Sable *et al*. 2006; Stromberg *et al*. 1998). Interestingly, adolescent rats were reported to be less tolerant to repeated dosing of naltrexone compared to adult rats in a 2-bottle choice ethanol paradigm (Sable *et al*. 2006). Therefore, future studies are necessary to examine chronic naltrexone dosing on MOR receptor regulation and operant ethanol self-administration during adolescence.

In our study, adolescent rats began operant ethanol self-administration at P36, which corresponds to early adolescence before the onset of puberty in male rats (Lewis *et al*. 2002; McCutcheon and Marinelli 2009; Spear 2000). This age was chosen to model epidemiological data reporting individuals that began drinking alcohol before the age of 14 were four times more likely to develop future alcohol dependence (DeWit *et al*. 2000; Grant and Dawson 1997). However, the firing rate of VTA dopamine neurons before pubertal onset is lower than in late adolescence around P45, when firing rate peaks (McCutcheon and Marinelli 2009). Adolescents are generally characterized to be more

sensitive to the rewarding properties and less sensitive to aversive properties of drugs of abuse including ethanol (Doremus-Fitzwater and Spear 2016). Therefore, it is possible the rewarding and aversive properties of ethanol may differ between the age of onset used in our study compared to if operant ethanol self-administration began during mid to late adolescence.

REGULATION OF EXTRACELLULAR GABA IN THE VTA

Our results are the first to report extracellular GABA concentration in the VTA measured using quantitative microdialysis. Functional differences in GABAergic transmission (Ikemoto 2007; Sanchez-Catalan *et al*. 2014) and ethanol reinforcement (Rodd-Henricks *et al*. 2000) are reported between VTA subregions. The basal extracellular GABA concentration in the current study (~45 nM) is largely represented by microdialysis probes located within the pVTA. Local microinjection of picrotoxin into the aVTA, but not the pVTA, increased dialysate dopamine concentration in the nucleus accumbens (Ding *et al*. 2009). These results suggest there may be increased GABAergic tone present in the aVTA. Therefore, we speculate heterogeneity in extracellular GABA concentration may also exist within the VTA, which could be dependent on GABAergic inputs and/or differences in GAT distribution. Future research should aim to determine the relative contribution of inhibitory inputs and GAT subtypes to extracellular GABA concentration following selective inactivation.

The efficiency of GATs is hypothesized to prevent changes in neuronal release of GABA from being detected by microdialysis in the extracellular space, which has

potential implications for interpreting whether drugs of abuse cause changes in extracellular GABA concentration. Indeed, our results further confirm extracellular GABA concentration in the VTA is highly regulated by GATs. Previous studies found local morphine delivered through the microdialysis probe caused a reduction in dialysate GABA concentration in the VTA (Klitenick *et al*. 1992; Sotomayor *et al*. 2005), but no effects were initially observed following systemic morphine administration (Ojanen *et al*. 2007). However, when GATs were inhibited using nipecotic acid, the increase in dialysate GABA concentration following nipecotic acid perfusion was reduced in animals that received an intravenous infusion of morphine (Vihavainen *et al*. 2008). Contrasting reports using *ex vivo* electrophysiological approaches show acute ethanol either increases (Theile *et al*. 2008; Theile *et al*. 2009; Theile *et al*. 2011) or decreases (Xiao *et al*. 2007; Xiao and Ye 2008) GABA release in the VTA, although the effect may also depend on subregion (Guan *et al*. 2012). In contrast, no changes in extracellular GABA measured by microdialysis have been observed following acute ethanol administration (Cowen *et al*. 1998; Kemppainen *et al*. 2010; Ojanen *et al*. 2007; Yan *et al*. 2005). Our current results suggest inhibiting GABA uptake prior to ethanol administration could be a useful strategy to determine whether acute ethanol results in changes to extracellular GABA concentration, which has potential to clarify the controversy between electrophysiological data using an *in vivo* model.

In contrast to acute ethanol administration, the effects of chronic ethanol consumption on extracellular GABA in the VTA are unexplored. Chronic ethanol has been shown to increase GABA neuron firing in the VTA (Gallegos *et al*. 1999) and basal

extracellular dopamine concentration in the pVTA was lower in rats following chronic ethanol consumption measured using quantitative microdialysis (Engleman *et al*. 2011). Chronic ethanol injections caused an elevation in basal dialysate GABA concentration in the nucleus accumbens (Dahchour *et al*. 1996), but no changes were detected during an acute ethanol challenge (Dahchour *et al*. 1994). Furthermore, higher levels of GAT-1 and GAT-3 were observed in the hippocampus of rats dependent on a liquid ethanol diet, although the VTA was not examined (Devaud 2001). Therefore, while GAT activity may prevent detection of acute changes in extracellular GABA, it is possible alterations in the regulation of basal extracellular GABA are implicated following chronic ethanol exposure.

Future experiments should also investigate whether GATs in the VTA may have a direct role in the maintenance of drug-dependent behaviors. Recently, GAT-1 was shown to enhance GABA release during opioid withdrawal in the periaqueductal gray (Bagley *et al*. 2011). GATs are also present on midbrain dopamine neurons to facilitate co-release of GABA (Tritsch *et al*. 2014). Interestingly, there is also clinical evidence showing tiagabine, a GAT-1 selective inhibitor, reduced alcohol withdrawal signs to a similar degree as benzodiazepines (Myrick *et al*. 2005). More studies are necessary to determine whether *in vivo* extraction fraction can be used as an accurate index of GABA clearance. However, quantitative microdialysis may prove to be a useful technique to examine functional changes in GAT activity during acute or chronic drug exposure.

Together, the results of the current experiments provide the foundation to further investigate the interaction between ethanol, the opioid peptide system and extracellular GABA concentration within the VTA.

References

Altshuler H. L., Phillips P. E., Feinhandler D. A. (1980) Alteration of ethanol self-administration by naltrexone. *Life Sci*. **26**, 679–88.

Anderzhanova E., Wotjak C. T. (2013) Brain microdialysis and its applications in experimental neurochemistry. *Cell Tissue Res*. **354**, 27–39.

Badanich K. A., Adler K. J., Kirstein C. L. (2006) Adolescents differ from adults in cocaine conditioned place preference and cocaine-induced dopamine in the nucleus accumbens septi. *Eur. J. Pharmacol*. **550**, 95–106.

Bagley E. E., Hacker J., Chefer V. I., Mallet C., McNally G. P., Chieng B. C. H., Perroud J., Shippenberg T. S., Christie M. J. (2011) Drug-induced GABA transporter currents enhance GABA release to induce opioid withdrawal behaviors. *Nat. Neurosci*. **14**, 1548–54.

Bankson M. G., Yamamoto B. K. (2004) Serotonin-GABA interactions modulate MDMA-induced mesolimbic dopamine release. *J. Neurochem*. **91**, 852–9.

Bell R. L., Rodd Z. A., Smith R. J., Toalston J. E., Franklin K. M., McBride W. J. (2011) Modeling binge-like ethanol drinking by peri-adolescent and adult P rats. *Pharmacol. Biochem. Behav*. **100**, 90–97.

Benveniste H., Hansen A. J., Ottosen N. S. (1989) Determination of brain interstitial concentrations by microdialysis. *J. Neurochem*. **52**, 1741–50.

Bocklisch C., Pascoli V., Wong J. C. Y., House D. R. C., Yvon C., DeRoo M., Tan K. R., Lüscher C. (2013) Cocaine disinhibits dopamine neurons by potentiation of GABA

transmission in the ventral tegmental area. *Science* **341**, 1521–5.

Bongiovanni R., Yamamoto B. K., Jaskiw G. E. (2001) Improved method for the measurement of large neutral amino acids in biological matrices. *J. Chromatogr. B Biomed. Sci. Appl.* **754**, 369–76.

Borden L. A. (1996) GABA transporter heterogeneity: Pharmacology and cellular localization. *Neurochem. Int.* **29**, 335–56.

Bourdelais A., Kalivas P. W. (1991) High sensitivity HPLC assay for GABA in brain dialysis studies. *J. Neurosci. Methods* **39**, 115–21.

Bowen E., West K. (1955) Solvent quenching of the fluorescence of anthracene. *J Chem Soc*, 4394–95.

Boyd B. W., Kennedy R. T. (1998) Determination of trace level gamma-aminobutyric acid using an improved OPA pre-column derivatization and on-column preconcentration capillary liquid chromatography with electrochemical detection. *Analyst* **123**, 2119–24.

Boyle A. E., Stewart R. B., Macenski M. J., Spiga R., Johnson B. A., Meisch R. A. (1998) Effects of acute and chronic doses of naltrexone on ethanol self-administration in rhesus monkeys. *Alcohol. Clin. Exp. Res.* **22**, 359–366.

Broadwater M., Varlinskaya E. I., Spear L. P. (2011) Chronic intermittent ethanol exposure in early adolescent and adult male rats: Effects on tolerance, social behavior, and ethanol intake. *Alcohol. Clin. Exp. Res.* **35**, 1392–1403.

Brodie M. S., Pesold C., Appel S. B. (1999) Ethanol directly excites dopaminergic ventral tegmental area reward neurons. *Alcohol. Clin. Exp. Res.* **23**, 1848–52.

Brodie M. S., Shefner S. A., Dunwiddie T. V (1990) Ethanol increases the firing rate of dopamine neurons of the rat ventral tegmental area in vitro. *Brain Res* **508**, 65–69.

Bröer S., Gether U. (2012) The solute carrier 6 family of transporters. *Br. J. Pharmacol.* **167**, 256–78.

Buczynski M. W., Herman M. A., Hsu K.-L., Natividad L. A., Irimia C., Polis I. Y., Pugh H., et al. (2016) Diacylglycerol lipase disinhibits VTA dopamine neurons during chronic nicotine exposure. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 1086–91.

Bungay P. M., Morrison P. F., Dedrick R. L. (1990) Steady-state theory for quantitative microdialysis of solutes and water in vivo and in vitro. *Life Sci.* **46**, 105–19.

Bungay P. M., Newton-Vinson P., Isele W., Garris P. A., Justice J. B. (2003) Microdialysis of dopamine interpreted with quantitative model incorporating probe implantation trauma. *J. Neurochem.* **86**, 932–46.

Burattini C., Gill T. M., Aicardi G., Janak P. H. (2006) The ethanol self-administration context as a reinstatement cue: acute effects of naltrexone. *Neuroscience* **139**, 877–87.

Burattini C., McGeehan A. J., Griffin W. C., Gass J. T., Kinder J. R., Janak P. H., Olive M. F. (2008) A microdialysis study of extracellular levels of acamprosate and naltrexone in the rat brain following acute and repeated administration. *Addict. Biol.* **13**, 70–9.

Carrillo J., Howard E. C., Moten M., Houck B. D., Czachowski C. L., Gonzales R. A. (2008) A three-day exposure to 10% ethanol with 10% sucrose successfully initiates ethanol self-administration. *Alcohol* **42**, 171–78.

Carter L. P., Griffiths R. R. (2009) Principles of laboratory assessment of drug abuse liability and implications for clinical development. *Drug Alcohol Depend*. **105 S1**, S14–S25.

Casey B. J., Jones R. M. (2010) Neurobiology of the adolescent brain and behavior: Implications for substance use disorders. *J. Am. Acad. Child Adolesc. Psychiatry* **49**, 1189–201.

Chefer V. I., Denoroy L., Zapata A., Shippenberg T. S. (2009a) Mu opioid receptor modulation of somatodendritic dopamine overflow: GABAergic and glutamatergic mechanisms. *Eur. J. Neurosci*. **30**, 272–8.

Chefer V. I., Thompson A. C., Zapata A., Shippenberg T. S. (2009b) Overview of brain microdialysis. *Curr. Protoc. Neurosci*. **47**, 7.1.1-7.1.28.

Chefer V., Meis J., Wang G., Kuzmin A., Bakalkin G., Shippenberg T. (2011) Repeated exposure to moderate doses of ethanol augments hippocampal glutamate neurotransmission by increasing release. *Addict. Biol*. **16**, 229–37.

Chen K. C. (2005) Evidence on extracellular dopamine level in rat striatum: implications for the validity of quantitative microdialysis. *J. Neurochem*. **92**, 46–58.

Chen R. F., Scott C., Trepman E. (1979) Fluorescence properties of o-phthaldialdehyde derivatives of amino acids. *Biochim Biophys Acta* **576**, 440–55.

Chotro M. G., Arias C. (2003) Prenatal exposure to ethanol increases ethanol consumption: a conditioned response? *Alcohol* **30**, 19–28.

Ciccocioppo R., Lin D., Martin-Fardon R., Weiss F. (2003) Reinstatement of ethanol-seeking behavior by drug cues following single versus multiple ethanol intoxication

in the rat: effects of naltrexone. *Psychopharmacology (Berl)*. **168**, 208–15.

Ciccocioppo R., Martin-Fardon R., Weiss F. (2002) Effect of selective blockade of mu(1) or delta opioid receptors on reinstatement of alcohol-seeking behavior by drug-associated stimuli in rats. *Neuropsychopharmacology* **27**, 391–9.

Cosford R. J., Parsons L. H., Justice J. B. (1994) Effect of tetrodotoxin and potassium infusion on microdialysis extraction fraction and extracellular dopamine in the nucleus accumbens. *Neurosci. Lett.* **178**, 175–78.

Cosford R. J., Vinson A. P., Kukoyi S., Justice J. B. (1996) Quantitative microdialysis of serotonin and norepinephrine: Pharmacological influences on in vivo extraction fraction. *J. Neurosci. Methods* **68**, 39–47.

Cowen M., Chen F., Jarrott B., Lawrence A. J. (1998) Effects of acute ethanol on GABA release and GABA(A) receptor density in the rat mesolimbic system. *Pharmacol. Biochem. Behav.* **59**, 51–7.

Cowen M. S., Rezvani A. H., Jarrott B., Lawrence A. J. (1999) Ethanol consumption by Fawn-Hooded rats following abstinence: effect of naltrexone and changes in mu-opioid receptor density. *Alcohol. Clin. Exp. Res.* **23**, 1008–14.

Creed M. C., Ntamati N. R., Tan K. R. (2014) VTA GABA neurons modulate specific learning behaviors through the control of dopamine and cholinergic systems. *Front. Behav. Neurosci.* **8**, 8.

Czachowski C. L., DeLory M. J. (2009) Acamprosate and naltrexone treatment effects on ethanol and sucrose seeking and intake in ethanol-dependent and nondependent rats. *Psychopharmacology (Berl)*. **204**, 335–48.

Dahchour A., Quertemont E., DeWitte P. (1994) Acute ethanol increases taurine but neither glutamate nor GABA in the nucleus accumbens of male rats: a microdialysis study. *Alcohol Alcohol*. **29**, 485–87.

Dahchour A., Quertemont E., DeWitte P. (1996) Taurine increases in the nucleus accumbens microdialysate after acute ethanol administration to naive and chronically alcoholised rats. *Brain Res*. **735**, 9–19.

Dalby N. O. (2000) GABA-level increasing and anticonvulsant effects of three different GABA uptake inhibitors. *Neuropharmacology* **39**, 2399–407.

Das S. C., Yamamoto B. K., Hristov A. M., Sari Y. (2015) Ceftriaxone attenuates ethanol drinking and restores extracellular glutamate concentration through normalization of GLT-1 in nucleus accumbens of male alcohol-preferring rats. *Neuropharmacology* **97**, 67–74.

de Freitas Silva D. M., Ferraz V. P., Ribeiro A. M. (2009) Improved high-performance liquid chromatographic method for GABA and glutamate determination in regions of the rodent brain. *J. Neurosci. Methods* **177**, 289–93.

De Groote L., Linthorst A. C. E. (2007) Exposure to novelty and forced swimming evoke stressor-dependent changes in extracellular GABA in the rat hippocampus. *Neuroscience* **148**, 794–805.

Del Arco A., Segovia G., Fuxe K., Mora F. (2003) Changes in dialysate concentrations of glutamate and GABA in the brain: an index of volume transmission mediated actions? *J. Neurochem*. **85**, 23–33.

Devaud L. L. (2001) Ethanol dependence has limited effects on GABA or glutamate transporters in rat brain. *Alcohol. Clin. Exp. Res.* **25**, 606–11.

DeWit D. J., Adlaf E. M., Offord D. R., Ogborne A. C. (2000) Age at first alcohol use: A risk factor for the development of alcohol disorders. *Am J Psychiatry* **157**, 745–50.

Ding Z.-M., Liu W., Engleman E. A., Rodd Z. A., McBride W. J. (2009) Differential effects of dopamine D2 and GABA(A) receptor antagonists on dopamine neurons between the anterior and posterior ventral tegmental area of female Wistar rats. *Pharmacol. Biochem. Behav.* **92**, 404–12.

Ding Z.-M., Rodd Z. A., Engleman E. A., Bailey J. A., Lahiri D. K., McBride W. J. (2013) Alcohol drinking and deprivation alter basal extracellular glutamate concentrations and clearance in the mesolimbic system of alcohol preferring (P) rats. *Addict. Biol.* **18**, 297–306.

Doherty J. M., Frantz K. J. (2012) Heroin self-administration and reinstatement of heroin-seeking in adolescent vs. adult male rats. *Psychopharmacology (Berl).* **219**, 763–73.

Doherty J. M., Gonzales R. A. (2015) Operant self-administration of sweetened ethanol and time course of blood ethanol levels in adolescent and adult male Long-Evans rats. *Alcohol. Clin. Exp. Res.* **39**, 485–95.

Doherty J., Ogbomnwan Y., Williams B., Frantz K. (2009) Age-dependent morphine intake and cue-induced reinstatement, but not escalation in intake, by adolescent and adult male rats. *Pharmacol. Biochem. Behav.* **92**, 164–72.

Donzanti B. A., Yamamoto B. K. (1988) An improved and rapid HPLC-EC method for

the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. *Life Sci*. **43**, 913–22.

Doremus-Fitzwater T. L., Spear L. P. (2016) Reward-centricity and attenuated aversions: An adolescent phenotype emerging from studies in laboratory animals. *Neurosci. Biobehav. Rev*. **70**, 121–34.

Doremus T. L., Brunell S. C., Rajendran P., Spear L. P. (2005) Factors influencing elevated ethanol consumption in adolescent relative to adult rats. *Alcohol. Clin. Exp. Res*. **29**, 1796–808.

Doyon W. M., Anders S. K., Ramachandra V. S., Czachowski C. L., Gonzales R. A. (2005) Effect of operant self-administration of 10% ethanol plus 10% sucrose on dopamine and ethanol concentrations in the nucleus accumbens. *J. Neurochem*. **93**, 1469–81.

Doyon W. M., York J. L., Diaz L. M., Samson H. H., Czachowski C. L., Gonzales R. A. (2003) Dopamine activity in the nucleus accumbens during consummatory phases of oral ethanol self-administration. *Alcohol. Clin. Exp. Res*. **27**, 1573–82.

Durkin M. M., Smith K. E., Borden L. A., Weinshank R. L., Branchek T. A., Gustafson E. L. (1995) Localization of messenger RNAs encoding three GABA transporters in rat brain: an in situ hybridization study. *Mol. Brain Res*. **33**, 7–21.

Durkin T. A., Anderson G. M., Cohen D. J. (1988) High-performance liquid chromatographic analysis of neurotransmitter amino acids in brain. *J. Chromatogr*. **428**, 9–15.

Ellgren M., Artmann A., Tkalych O., Gupta A., Hansen H. S., Hansen S. H., Devi L. A.,

Hurd Y. L. (2008) Dynamic changes of the endogenous cannabinoid and opioid mesocorticolimbic systems during adolescence: THC effects. *Eur. Neuropsychopharmacol.* **18**, 826–34.

Engleman E. A., Keen E. J., Tilford S. S., Thielen R. J., Morzorati S. L. (2011) Ethanol drinking reduces extracellular dopamine levels in the posterior ventral tegmental area of nondependent alcohol-preferring rats. *Alcohol* **45**, 549–57.

Fields H. L., Hjelmstad G. O., Margolis E. B., Nicola S. M. (2007) Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu. Rev. Neurosci.* **30**, 289–316.

Fliegel S., Brand I., Spanagel R., Noori H. R. (2013) Ethanol-induced alterations of amino acids measured by in vivo microdialysis in rats: a meta-analysis. *Silico Pharmacol.* **1**, 7.

Ford C. P., Mark G. P., Williams J. T. (2006) Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location. *J. Neurosci.* **26**, 2788–97.

Frantz K. J., O'Dell L. E., Parsons L. H. (2007) Behavioral and neurochemical responses to cocaine in periadolescent and adult rats. *Neuropsychopharmacology* **32**, 625–637.

Froehlich J. C., Harts J., Lumeng L., Li T. K. (1990) Naloxone attenuates voluntary ethanol intake in rats selectively bred for high ethanol preference. *Pharmacol. Biochem. Behav.* **35**, 385–90.

Gallegos R. A., Lee R.-S., Criado J. R., Henriksen S. J., Steffensen S. C. (1999) Adaptive responses of γ-aminobutyric acid neurons in the ventral tegmental area to chronic

ethanol. *J. Pharmacol. Exp. Ther.* **291**, 1045–53.

García-Burgos D., González F., Manrique T., Gallo M. (2009) Patterns of ethanol intake in preadolescent, adolescent, and adult wistar rats under acquisition, maintenance, and relapse-like conditions. *Alcohol. Clin. Exp. Res.* **33**, 722–28.

Garzon M., Pickel V. M. (2001) Plasmalemmal mu-opioid receptor distribution mainly in nondopaminergic neurons in the rat ventral tegmental area. *Synapse* **41**, 311–28.

Georges F., Normand E., Bloch B., Moine C. Le (1998) Opioid receptor gene expression in the rat brain during ontogeny, with special reference to the mesostriatal system: an in situ hybridization study. *Dev. Brain Res.* **109**, 187–99.

Gessa G. L., Muntoni F., Collu M., Varqui L., Mereu G. (1985) Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. *Brain Res.* **348**, 201–3.

Gonzales R. A., Job M. O., Doyon W. M. (2004) The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. *Pharmacol. Ther.* **103**, 121–46.

Gonzales R. A., Weiss F. (1998) Suppression of ethanol-reinforced behavior by naltrexone is associated with attenuation of the ethanol-induced increase in dialysate dopamine levels in the nucleus accumbens. *J. Neurosci.* **18**, 10663–71.

González G., Desai R., Sofuoglu M., Poling J., Oliveto A., Gonsai K., Kosten T. R. (2007) Clinical efficacy of gabapentin versus tiagabine for reducing cocaine use among cocaine dependent methadone-treated patients. *Drug Alcohol Depend.* **87**, 1–9.

Grant B. F., Dawson D. A. (1997) Age at onset of alcohol use and its association with

DSM-IV alcohol abuse and dependence: results from the national longitudinal alcohol epidemiologic survey. *J. Subst. Abuse* **9**, 103–10.

Grant B., Goldstein R., Saha T., Chou P. S., Jung J., Zhang H., Pickering R., et al. (2015) Epidemiology of DSM-5 alcohol use disorder: Results from the National Epidemiologic Survey on Alcohol and Related Conditions III. *JAMA Psychiatry* **72**, 757–66.

Guan Y., Xiao C., Krnjevic K., Xie G., Zuo W., Ye J.-H. (2012) GABAergic actions mediate opposite ethanol effects on dopaminergic neurons in the anterior and posterior ventral tegmental area. *J. Pharmacol. Exp. Ther.* **341**, 33–42.

Hall F. S., Sora I., Uhl G. R. (2001) Ethanol consumption and reward are decreased in mu-opiate receptor knockout mice. *Psychopharmacology (Berl).* **154**, 43–9.

Hallmark R. A., Hunt P. S. (2004) Social learning about ethanol in preweanling rats: role of endogenous opioids. *Dev. Psychobiol.* **44**, 132–9.

Hay R. A., Jennings J. H., Zitzman D. L., Hodge C. W., Robinson D. L. (2013) Specific and nonspecific effects of naltrexone on goal-directed and habitual models of alcohol seeking and drinking. *Alcohol. Clin. Exp. Res.* **37**, 1100–10.

Henderson-Redmond A., Czachowski C. (2014) Effects of systemic opioid receptor ligands on ethanol- and sucrose seeking and drinking in alcohol-preferring (P) and Long Evans rats. *Psychopharmacology (Berl).* **231**, 4309–21.

Herz A. (1997) Endogenous opioid systems and alcohol addiction. *Psychopharmacology (Berl).* **129**, 99–111.

Hingson R. W., Heeren T., Winter M. R. (2006) Age at drinking onset and alcohol

dependence: age at onset, duration, and severity. *Arch. Pediatr. Adolesc. Med.* **160**, 739–46.

Hodge C. W., Haraguchi M., Erickson H., Samson H. H. (1993) Ventral tegmental microinjections of quinpirole decrease ethanol and sucrose-reinforced responding. *Alcohol. Clin. Exp. Res.* **17**, 370–75.

Ikemoto S. (2005) The supramammillary nucleus mediates primary reinforcement via GABAA receptors. *Neuropsychopharmacology* **30**, 1088–95.

Ikemoto S. (2007) Dopamine reward circuitry: Two projection systems from the ventral midbrain to the nucleus accumbens–olfactory tubercle complex. *Brain Res. Rev.* **56**, 27–78.

Ikemoto S., Murphy J. M., McBride W. J. (1997) Self-infusion of GABA(A) antagonists directly into the ventral tegmental area and adjacent regions. *Behav. Neurosci.* **111**, 369–80.

Jacobs W. A. (1987) o-Phthalaldehyde—sulfite derivaization of primary amines for liquid chromatography—electrochemistry. *J. Chromatogr. A* **392**, 435–41.

Jalabert M., Bourdy R., Courtin J., Veinante P., Manzoni O. J., Barrot M., Georges F. (2011) Neuronal circuits underlying acute morphine action on dopamine neurons. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 16446–50.

Jarjour S., Bai L., Gianoulakis C. (2009) Effect of acute ethanol administration on the release of opioid peptides from the midbrain including the ventral tegmental area. *Alcohol. Clin. Exp. Res.* **33**, 1033–43.

Jhou T. C., Geisler S., Marinelli M., Degarmo B. A., Zahm D. S. (2009) The

mesopontine rostromedial tegmental nucleus: A structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. *J. Comp. Neurol.* **513**, 566–96.

Johnson S. W., North R. A. (1992) Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J. Neurosci.* **12**, 483–8.

Johnston G. A., Stephanson A. L., Twitchin B. (1976) Uptake and release of nipecotic acid by rat brain slices. *J. Neurochem.* **26**, 83–7.

June H. L., Cummings R., Eiler W. J. A. 2nd, Foster K. L., McKay P. F., Seyoum R., Garcia M., et al. (2004) Central opioid receptors differentially regulate the nalmefene-induced suppression of ethanol- and saccharin-reinforced behaviors in alcohol-preferring (P) rats. *Neuropsychopharmacology* **29**, 285–99.

Justice J. B. (1993) Quantitative microdialysis of neurotransmitters. *J. Neurosci. Methods* **48**, 263–76.

Katner S. N., Magalong J. G., Weiss F. (1999) Reinstatement of alcohol-seeking behavior by drug-associated discriminative stimuli after prolonged extinction in the rat. *Neuropsychopharmacology* **20**, 471–79.

Kehr J. (1993) *Fluorescence detection of amino acids derivatized with o-phthalaldehyde (OPA) based reagents*. Stockholm, Sweden.

Kehr J. (1998) Determination of γ-aminobutyric acid in microdialysis samples by microbore column liquid chromatography and fluorescence detection. *J. Chromatogr. B Biomed. Sci. Appl.* **708**, 49–54.

Kemppainen H., Raivio N., Nurmi H., Kiianmaa K. (2010) GABA and glutamate

overflow in the VTA and ventral pallidum of alcohol-preferring AA and alcohol-avoiding ANA rats after ethanol. *Alcohol Alcohol*. **45**, 111–8.

Kenakin T. (1997) Statistical assessment of biological significance from data, in *Pharmacol. Anal. Drug-Receptor Interact*., pp. 198–241. Lippincott-Raven, Philadelphia.

Kersanté F., Rowley S. C. S., Pavlov I., Gutièrrez-Mecinas M., Semyanov A., Reul J. M. H. M., Walker M. C., Linthorst A. C. E. (2013) A functional role for both γ-aminobutyric acid (GABA) transporter-1 and GABA transporter-3 in the modulation of extracellular GABA and GABAergic tonic conductances in the rat hippocampus. *J. Physiol*. **591**, 2429–41.

Klitenick M. A., DeWitte P., Kalivas P. W. (1992) Regulation of somatodendritic dopamine release in the ventral tegmental area by opioids and GABA: an in vivo microdialysis study. *J. Neurosci*. **12**, 2623–32.

Koob G. F., Volkow N. D. (2010) Neurocircuitry of addiction. *Neuropsychopharmacology* **35**, 217–38.

Krogsgaard-Larsen P., Falch E., Larsson O. M., Schousboe A. (1987) GABA uptake inhibitors: relevance to antiepileptic drug research. *Epilepsy Res*. **1**, 77–93.

Krystal J. H., Cramer J. A., Krol W. F., Kirk G. F., Rosenheck R. A. (2001) Naltrexone in the treatment of alcohol dependence. *N. Engl. J. Med*. **345**, 1734–39.

Lasek A., Janak P., He L., Whistler J., Heberlein U. (2007) Downregulation of mu opioid receptor by RNA interference in the ventral tegmental area reduces ethanol consumption in mice. *Genes. Brain. Behav*. **6**, 728–35.

Lasley S. M., Greenland R. D., Michaelson I. A. (1984) Determination of gamma-aminobutyric and glutamic acids in rat brain by liquid chromatography with electrochemical detection. *Life Sci*. **35**, 1921–30.

Lecca S., Melis M., Luchicchi A., Ennas M. G., Castelli M. P., Muntoni A. L., Pistis M. (2011) Effects of drugs of abuse on putative rostromedial tegmental neurons, inhibitory afferents to midbrain dopamine cells. *Neuropsychopharmacology* **36**, 589–602.

Lecca S., Melis M., Luchicchi A., Muntoni A. L., Pistis M. (2012) Inhibitory inputs from rostromedial tegmental neurons regulate spontaneous activity of midbrain dopamine cells and their responses to drugs of abuse. *Neuropsychopharmacology* **37**, 1164–76.

Lesscher H. M. B., Bailey A., Burbach J. P. H., Ree J. M. Van, Kitchen I., Gerrits M. A. F. M. (2003) Receptor-selective changes in mu-, delta- and kappa-opioid receptors after chronic naltrexone treatment in mice. *Eur. J. Neurosci*. **17**, 1006–12.

Lewis E. M., Barnett J. F., Freshwater L., Hoberman A. M., Christian M. S. (2002) Sexual maturation data for Crl Sprague-Dawley rats: Criteria and confounding factors. *Drug Chem. Toxicol*. **25**, 437–58.

Li C., Frantz K. J. (2009) Attenuated incubation of cocaine seeking in male rats trained to self-administer cocaine during periadolescence. *Psychopharmacology (Berl)*. **204**, 725–33.

Li P., Wu P., Xin X., Fan Y.-L., Wang G.-B., Wang F., Ma M.-Y., et al. (2015) Incubation of alcohol craving during abstinence in patients with alcohol dependence. *Addict. Biol*. **20**, 513–22.

Lindroth P., Mopper K. (1979) High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phthaldialdehyde. *Anal. Chem.* **51**, 1667–74.

Litten R. Z., Fertig J., Mattson M., Egli M. (2005) Development of medications for alcohol use disorders: recent advances and ongoing challenges. *Expert Opin. Emerg. Drugs* **10**, 323–43.

Lonnroth P., Jansson P. A., Smith U. (1987) A microdialysis method allowing characterization of intercellular water space in humans. *Am. J. Physiol.* **253**, E228-31.

Lüscher C., Malenka R. C. (2011) Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. *Neuron* **69**, 650–63.

Madsen K. K., White H. S., Schousboe A. (2010) Neuronal and non-neuronal GABA transporters as targets for antiepileptic drugs. *Pharmacol. Ther.* **125**, 394–401.

Mansour A., Fox C. A., Akil H., Watson S. J. (1995) Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* **18**, 22–9.

Mansour A., Fox C. A., Thompson R. C., Akil H., Watson S. J. (1994) mu-Opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. *Brain Res.* **643**, 245–65.

Margolis E. B., Hjelmstad G. O., Bonci A., Fields H. L. (2003) Kappa-opioid agonists directly inhibit midbrain dopaminergic neurons. *J. Neurosci.* **23**, 9981–6.

Margolis E. B., Lock H., Chefer V. I., Shippenberg T. S., Hjelmstad G. O., Fields H. L. (2006a) Kappa opioids selectively control dopaminergic neurons projecting to the

prefrontal cortex. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2938–2942.

Margolis E. B., Lock H., Hjelmstad G. O., Fields H. L. (2006b) The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? *J. Physiol.* **577**, 907–24.

Margolis E. B., Mitchell J. M., Ishikawa J., Hjelmstad G. O., Fields H. L. (2008) Midbrain dopamine neurons: projection target determines action potential duration and dopamine D2 receptor inhibition. *J. Neurosci.* **28**, 8908–13.

Margolis E. B., Toy B., Himmels P., Morales M., Fields H. L. (2012) Identification of rat ventral tegmental area GABAergic neurons. *PLoS One* **7**, e42365.

Matsui A., Jarvie B. C., Robinson B. G., Hentges S. T., Williams J. T. (2014) Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal. *Neuron* **82**, 1346–56.

Matsui A., Williams J. T. (2011) Opioid-sensitive GABA inputs from rostromedial tegmental nucleus synapse onto midbrain dopamine neurons. *J. Neurosci.* **31**, 17729–35.

McCutcheon J. E., Conrad K. L., Carr S. B., Ford K. A., McGehee D. S., Marinelli M. (2012) Dopamine neurons in the ventral tegmental area fire faster in adolescent rats than in adults. *J. Neurophysiol.* **108**, 1620–30.

McCutcheon J. E., Marinelli M. (2009) Age matters. *Eur. J. Neurosci.* **29**, 997–1014.

Meldrum B. S., Rogawski M. A. (2007) Molecular Targets for Antiepileptic Drug Development. *Neurotherapeutics* **4**, 18–61.

Melendez R. I., Hicks M. P., Cagle S. S., Kalivas P. W. (2005) Ethanol exposure

decreases glutamate uptake in the nucleus accumbens. *Alcohol. Clin. Exp. Res.* **29**, 326–33.

Melis M., Camarini R., Ungless M. a, Bonci A. (2002) Long-lasting potentiation of GABAergic synapses in dopamine neurons after a single in vivo ethanol exposure. *J. Neurosci.* **22**, 2074–82.

Middaugh L. D., Szumlinski K. K., Patten Y. Van, Marlowe A.-L. B., Kalivas P. W. (2003) Chronic ethanol consumption by C57BL/6 mice promotes tolerance to its interoceptive cues and increases extracellular dopamine, an effect blocked by naltrexone. *Alcohol. Clin. Exp. Res.* **27**, 1892–1900.

Miranda R., Ray L., Blanchard A., Reynolds E. K., Monti P. M., Chun T., Justus A., et al. (2014) Effects of naltrexone on adolescent alcohol cue reactivity and sensitivity: an initial randomized trial. *Addict. Biol.* **19**, 941–54.

Miranda R., Treloar H. (2016) Emerging pharmacologic treatments for adolescent substance use: challenges and new directions. *Curr. Addict. Reports* **3**, 145–56.

Mitchell J. M., Bergren L. J., Chen K. S., Rowbotham M. C., Fields H. L. (2009) Naltrexone aversion and treatment efficacy are greatest in humans and rats that actively consume high levels of alcohol. *Neurobiol. Dis.* **33**, 72–80.

Molnár-Perl I., Bozor I. (1998) Comparison of the stability and UV and fluorescence characteristics of the o-phthaldialdehyde/3-mercaptopropionic acid and o-phthaldialdehyde/N-acetyl-l-cysteine reagents and those of their amino acid derivatives. *J. Chromatogr. A* **798**, 37–46.

Morrison P. F., Bungay P. M., Hsiao J. K., Ball B. A., Mefford I. N., Dedrick R. L.

(1991) Quantitative microdialysis: analysis of transients and application to pharmacokinetics in brain. *J. Neurochem.* **57**, 103–19.

Myers R. D., Borg S., Mossberg R. (1986) Antagonism by naltrexone of voluntary alcohol selection in the chronically drinking macaque monkey. *Alcohol* **3**, 383–388.

Myrick H., Taylor B., LaRowe S., Nguyen S., Boyle E., Cochran K., Malcolm R. (2005) A retrospective chart review comparing tiagabine and benzodiazepines for the treatment of alcohol withdrawal. *J. Psychoactive Drugs* **37**, 409–14.

Nair-Roberts R. G., Chatelain-Badie S. D., Benson E., White-Cooper H., Bolam J. P., Ungless M. A. (2008) Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. *Neuroscience* **152**, 1024–31.

Nance D. M. (1983) The developmental and neural determinants of the effects of estrogen on feeding behavior in the rat: A theoretical perspective. *Neurosci. Biobehav. Rev.* **7**, 189–211.

Natividad L. A., Buczynski M. W., Parsons L. H., Torres O. V, O'Dell L. E. (2012) Adolescent rats are resistant to adaptations in excitatory and inhibitory mechanisms that modulate mesolimbic dopamine during nicotine withdrawal. *J. Neurochem.* **123**, 578–88.

Natividad L., Tejeda H., Torres O., O'Dell L. (2010) Nicotine withdrawal produces a decrease in extracellular levels of dopamine in the nucleus accumbens that is lower in adolescent versus adult male rats. *Synapse* **64**, 136–145.

Nugent F. S., Kauer J. A. (2008) LTP of GABAergic synapses in the ventral tegmental

area and beyond. *J. Physiol.* **586**, 1487–93.

Nyitrai G., Kékesi K. A., Juhász G. (2006) Extracellular level of GABA and Glu: in vivo microdialysis-HPLC measurements. *Curr. Top. Med. Chem.* **6**, 935–40.

O'Dell L. E., Parsons L. H. (2004) Serotonin1B receptors in the ventral tegmental area modulate cocaine-induced increases in nucleus accumbens dopamine levels. *J. Pharmacol. Exp. Ther.* **311**, 711–19.

O'Malley S., Jaffe A., Chang G., Schottenfeld R., Meyer R., Rounsaville B. (1992) Naltrexone and coping skills therapy for alcohol dependence: a controlled study. *Arch. Gen. Psychiatry* **49**, 881–7.

O'Malley S. S., Corbin W. R., Leeman R. F., DeMartini K. S., Fucito L. M., Ikomi J., Romano D. M., et al. (2015) Reduction of Alcohol Drinking in Young Adults by Naltrexone: A Double-Blind, Placebo-Controlled, Randomized Clinical Trial of Efficacy and Safety. *J. Clin. Psychiatry* **76**, e207–e213.

Ojanen S. P., Palmén M., Hyytiä P., Kiianmaa K. (2007) Extracellular glutamate and GABA in the ventral tegmental area of alcohol-preferring AA and alcohol-avoiding ANA rats treated repeatedly with morphine. *Eur. J. Pharmacol.* **559**, 38–45.

Okamoto T., Harnett M. T., Morikawa H. (2006) Hyperpolarization-activated cation current (Ih) is an ethanol target in midbrain dopamine neurons of mice. *J. Neurophysiol.* **95**, 619–26.

Oliva I., Wanat M. J. (2016) Ventral tegmental area afferents and drug-dependent behaviors. *Front. Psychiatry* **7**, 30.

Olive M. F., Koenig H. N., Nannini M. A., Hodge C. W. (2001) Stimulation of endorphin

neurotransmission in the nucleus accumbens by ethanol, cocaine, and amphetamine. *J. Neurosci.* **21**, RC184.

Olson R., Justice J. (1993) Quantitative microdialysis under transient conditions. *Anal. Chem.* **65**, 1017–22.

Olson V. G., Zabetian C. P., Bolanos C. A., Edwards S., Barrot M., Eisch A. J., Hughes T., Self D. W., Neve R. L., Nestler E. J. (2005) Regulation of drug reward by cAMP response element-binding protein: evidence for two functionally distinct subregions of the ventral tegmental area. *J. Neurosci.* **25**, 5553–62.

Palm S., Nylander I. (2014) Alcohol-induced changes in opioid peptide levels in adolescent rats are dependent on housing conditions. *Alcohol. Clin. Exp. Res.* **38**, 2978–87.

Parsons L. H., Justice J. B. (1992) Extracellular Concentration and In Vivo Recovery of Dopamine in the Nucleus Accumbens Using Microdialysis. *J. Neurochem.* **58**, 212–18.

Parsons L. H., Smith A. D., Justice J. B. J. (1991) The in vivo microdialysis recovery of dopamine is altered independently of basal level by 6-hydroxydopamine lesions to the nucleus accumbens. *J. Neurosci. Methods* **40**, 139–47.

Pati D., Kelly K., Stennett B., Frazier C. J., Knackstedt L. A. (2016) Alcohol consumption increases basal extracellular glutamate in the nucleus accumbens core of Sprague-Dawley rats without increasing spontaneous glutamate release. *Eur. J. Neurosci.* **44**, 1896–905.

Paxinos G., Watson C. (2007) *The Rat Brain in Stereotaxic Coordinates*. Elsevier,

Amsterdam.

Peng X.-Q., Li X., Gilbert J. G., Pak A. C., Ashby C. R., Brodie J. D., Dewey S. L., Gardner E. L., Xi Z.-X. (2008) Gamma-vinyl GABA inhibits cocaine-triggered reinstatement of drug-seeking behavior in rats by a non-dopaminergic mechanism. *Drug Alcohol Depend*. **97**, 216–25.

Perucho J., Gonzalo-Gobernado R., Bazan E., Casarejos M. J., Jiménez-Escrig A., Asensio M. J., Herranz A. S. (2015) Optimal excitation and emission wavelengths to analyze amino acids and optimize neurotransmitters quantification using precolumn OPA-derivatization by HPLC. *Amino Acids* **47**, 963–73.

Pettit H. O., Justice J. B. (1991a) Effect of dose on cocaine self-administration behavior and dopamine levels in the nucleus accumbens. *Brain Res*. **539**, 94–102.

Pettit H. O., Justice J. J. (1991b) Procedures for microdialysis with small-bore HPLC, in *Microdialysis Neurosci*., (Robinson T., Justice J. J., eds), pp. 117–153. Elsevier, New York.

Philpot R. M., Wecker L., Kirstein C. L. (2009) Repeated ethanol exposure during adolescence alters the developmental trajectory of dopaminergic output from the nucleus accumbens septi. *Int. J. Dev. Neurosci*. **27**, 805–815.

Piepponen T. P., Skujins A. (2001) Rapid and sensitive step gradient assays of glutamate, glycine, taurine and γ-aminobutyric acid by high-performance liquid chromatography–fluorescence detection with o-phthalaldehyde–mercaptoethanol derivatization with an emphasis on microdialysis samples. *J. Chromatogr. B Biomed. Sci. Appl*. **757**, 277–83.

Rea K., Cremers T. I. F. H., Westerink B. H. C. (2005) HPLC conditions are critical for the detection of GABA by microdialysis. *J. Neurochem.* **94**, 672–9.

Reichel C. M., Bevins R. A. (2009) Forced abstinence model of relapse to study pharmacological treatments of substance use disorder. *Curr. Drug Abuse Rev.* **2**, 184–94.

Reinhoud N. J., Brouwer H.-J., Heerwaarden L. M. van, Korte-Bouws G. A. H. (2013) Analysis of glutamate, GABA, noradrenaline, dopamine, serotonin, and metabolites using microbore UHPLC with electrochemical detection. *ACS Chem. Neurosci.* **4**, 888–94.

Richard J. M., Fields H. L. (2016) Mu-opioid receptor activation in the medial shell of nucleus accumbens promotes alcohol consumption, self-administration and cue-induced reinstatement. *Neuropharmacology* **108**, 14–23.

Roberts A. J., McDonald J. S., Heyser C. J., Kieffer B. L., Matthes H. W. D., Koob G. F., Gold L. H. (2000) μ-Opioid receptor knockout mice do not self-administer alcohol. *J. Pharmacol. Exp. Ther.* **293**, 1002–8.

Rodd-Henricks A. Z., McKinzie L. D., Crile S. R., Murphy M. J., McBride J. W. (2000) Regional heterogeneity for the intracranial self-administration of ethanol within the ventral tegmental area of female Wistar rats. *Psychopharmacology (Berl).* **149**, 217–24.

Rodd Z. A., Bell R. L., Zhang Y., Murphy J. M., Goldstein A., Zaffaroni A., Li T.-K., McBride W. J. (2004) Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring

(P) rats: involvement of dopamine and serotonin. *Neuropsychopharmacology* **30**, 330–38.

Rosner S., Hackl-Herrwerth A., Leucht S., Lehert P., Vecchi S., Soyka M. (2010) Acamprosate for alcohol dependence. *Cochrane Database Syst. Rev.* 9, CD004332.

Roth F. C., Draguhn A. (2012) GABA Metabolism and Transport: Effects on Synaptic Efficacy. *Neural Plast.* **2012**, 805830.

Roth M. (1971) Fluorescence reaction for amino acids. *Anal. Chem.* **43**, 880–82.

Rowley H. L., Martin K. F., Marsden C. A. (1995) Determination of in vivo amino acid neurotransmitters by high-performance liquid chromatography with o-phthalaldehyde-sulphite derivatisation. *J. Neurosci. Methods* **57**, 93–9.

Sabino V., Kwak J., Rice K. C., Cottone P. (2013) Pharmacological characterization of the 20% alcohol intermittent access model in Sardinian alcohol-preferring rats: a model of binge-like drinking. *Alcohol. Clin. Exp. Res.* **37**, 635–43.

Sable H. J. K., Bell R. L., Rodd Z. A., McBride W. J. (2006) Effects of naltrexone on the acquisition of alcohol intake in male and female periadolescent and adult alcohol-preferring (P) rats. *Int. J. Adolesc. Med. Health* **18**, 139–49.

Sacks J. J., Gonzales K. R., Bouchery E. E., Tomedi L. E., Brewer R. D. (2015) 2010 National and State Costs of Excessive Alcohol Consumption. *Am. J. Prev. Med.* **49**, e73-9.

SAMHSA (2015) *Results from the 2014 National Survey on Drug Use and Health: Summary of National Findings*. Substance Abuse and Mental Health Services Administration, Rockville, MD.

Sanchez-Catalan M. J., Kaufling J., Georges F., Veinante P., Barrot M. (2014) The antero-posterior heterogeneity of the ventral tegmental area. *Neuroscience* **282**, 198–216.

Schifirneţ E., Bowen S. E., Borszcz G. S. (2014) Separating analgesia from reward within the ventral tegmental area. *Neuroscience* **263**, 72–87.

Schindler A. G., Soden M. E., Zweifel L. S., Clark J. J. (2016) Reversal of alcohol-induced dysregulation in dopamine network dynamics may rescue maladaptive decision-making. *J. Neurosci.* **36**, 3698–708.

Schindler A. G., Tsutsui K. T., Clark J. J. (2014) Chronic alcohol intake during adolescence, but not adulthood, promotes persistent deficits in risk-based decision making. *Alcohol. Clin. Exp. Res.* **38**, 1622–29.

Schousboe A., Bak L., Waagepetersen H. (2013) Astrocytic control of biosynthesis and turnover of the neurotransmitters glutamate and GABA. *Front. Endocrinol. (Lausanne).* **4**, 102.

Schramm-Sapyta N. L., DiFeliceantonio A. G., Foscue E., Glowacz S., Haseeb N., Wang N., Zhou C., Kuhn C. M. (2010) Aversive effects of ethanol in adolescent versus adult rats: potential causes and implication for future drinking. *Alcohol. Clin. Exp. Res.* **34**, 2061–69.

Scimemi A. (2014) Structure, function, and plasticity of GABA transporters. *Front. Cell. Neurosci.* **8**, 161.

Shah A. J., Crespi F., Heidbreder C. (2002) Amino acid neurotransmitters: separation approaches and diagnostic value. *J. Chromatogr. B* **781**, 151–63.

Shen H., Scofield M. D., Boger H., Hensley M., Kalivas P. W. (2014) Synaptic glutamate spillover due to impaired glutamate uptake mediates heroin relapse. *J. Neurosci.* **34**, 5649–57.

Siegmund S., Vengeliene V., Singer M. V, Spanagel R. (2005) Influence of age at drinking onset on long-term ethanol self-administration with deprivation and stress phases. *Alcohol. Clin. Exp. Res.* **29**, 1139–45.

Simons S. S., Johnson D. F. (1978) Reaction of o-phthalaldehyde and thiols with primary amines: Fluorescence properties of 1-alkyl(and aryl)thio-2-alkylisoindoles. *Anal. Biochem.* **90**, 705–25.

Sinclair J. D. (2001) Evidence about the use of naltrexone and for different ways of using it in the treatment of alcoholism. *Alcohol Alcohol.* **36**, 2–10.

Slaney T. R., Mabrouk O. S., Porter-Stransky K. a, Aragona B. J., Kennedy R. T. (2013) Chemical gradients within brain extracellular space measured using low flow push-pull perfusion sampling in vivo. *ACS Chem. Neurosci.* **4**, 321–9.

Smith A. D., Justice J. B. (1994) The effect of inhibition of synthesis, release, metabolism and uptake on the microdialysis extraction fraction of dopamine. *J. Neurosci. Methods* **54**, 75–82.

Smith S., Sharp T. (1994) Measurement of GABA in rat brain microdialysates using o-phthaldialdehyde—sulphite derivatization and high-performance liquid chromatography with electrochemical detection. *J. Chromatogr. B Biomed. Sci. Appl.* **652**, 228–33.

Sofuoglu M., Mouratidis M., Yoo S., Culligan K., Kosten T. (2005) Effects of tiagabine

in combination with intravenous nicotine in overnight abstinent smokers. *Psychopharmacology (Berl)*. **181**, 504–10.

Solís J. M., Nicoll R. A. (1992) Postsynaptic action of endogenous GABA released by nipecotic acid in the hippocampus. *Neurosci. Lett*. **147**, 16–20.

Sotomayor-Zarate R., Abarca J., Araya K. A., Renard G. M., Andrés M. E., Gysling K. (2015) Exposure to repeated immobilization stress inhibits cocaine-induced increase in dopamine extracellular levels in the rat ventral tegmental area. *Pharmacol. Res*. **101**, 116–23.

Sotomayor R., Forray M. I., Gysling K. (2005) Acute morphine administration increases extracellular DA levels in the rat lateral septum by decreasing the GABAergic inhibitory tone in the ventral tegmental area. *J. Neurosci. Res*. **81**, 132–9.

Spain J. W., Roth B. L., Coscia C. J. (1985) Differential ontogeny of multiple opioid receptors (mu, delta, and kappa). *J. Neurosci*. **5**, 584–8.

Spanagel R., Herz A., Shippenberg T. S. (1992) Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proc. Natl. Acad. Sci. U. S. A*. **89**, 2046–50.

Spanagel R., Vengeliene V., Jandeleit B., Fischer W.-N., Grindstaff K., Zhang X., Gallop M. A., Krstew E. V, Lawrence A. J., Kiefer F. (2014) Acamprosate produces its anti-relapse effects via calcium. *Neuropsychopharmacology* **39**, 783–91.

Spear L. P. (2000) The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev*. **24**, 417–63.

Steffensen S. C., Walton C. H., Hansen D. M., Yorgason J. T., Gallegos R. A., Criado J.

R. (2009) Contingent and non-contingent effects of low-dose ethanol on GABA neuron activity in the ventral tegmental area. *Pharmacol. Biochem. Behav.* **92**, 68–75.

Stobbs S. H., Ohran A. J., Lassen M. B., Allison D. W., Brown J. E., Steffensen S. C. (2004) Ethanol suppression of ventral tegmental area GABA neuron electrical transmission involves N-methyl-D-aspartate receptors. *J. Pharmacol. Exp. Ther.* **311**, 282–89.

Stromberg M. F., Volpicelli J. R., O'Brien C. P. (1998) Effects of naltrexone administered repeatedly across 30 or 60 days on ethanol consumption using a limited access procedure in the rat. *Alcohol. Clin. Exp. Res.* **22**, 2186–2191.

Stuber G. D., Hnasko T. S., Britt J. P., Edwards R. H., Bonci A. (2010) Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. *J. Neurosci.* **30**, 8229–33.

Suzuki S., Fujii T., Imai A., Akahori H. (1977) The fluorescent level inversion of dual fluorescences and the motional relaxation of excited state molecules in solutions. *J. Phys. Chem.* **81**, 1592–98.

Svingos A. L., Garzon M., Colago E. E., Pickel V. M. (2001) Mu-opioid receptors in the ventral tegmental area are targeted to presynaptically and directly modulate mesocortical projection neurons. *Synapse* **41**, 221–9.

Swanson L. W. (1982) The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res. Bull.* **9**, 321–53.

Swendsen J., Burstein M., Case B., Conway K. P., Dierker L., He J., Merikangas K. R. (2012) Use and abuse of alcohol and illicit drugs in US adolescents: results of the National Comorbidity Survey–Adolescent Supplement. *Arch. Gen. Psychiatry* **69**, 390–98.

Szerb J. C. (1982) Effect of nipecotic acid, a γ-aminobutyric acid transport inhibitor, on the turnover and release of γ-aminobutyric acid in rat cortical slices. *J. Neurochem.* **39**, 850–8.

Talbot J. N., Happe H. K., Murrin L. C. (2005) μ ppioid receptor coupling to Gi/o proteins increases during postnatal development in rat brain. *J. Pharmacol. Exp. Ther.* **314**, 596–602.

Theile J., Morikawa H., Gonzales R., Morrisett R. (2011) GABAergic transmission modulates ethanol excitation of ventral tegmental area dopamine neurons. *Neuroscience* **6088**, 94–103.

Theile J. W., Morikawa H., Gonzales R. A., Morrisett R. A. (2008) Ethanol enhances GABAergic transmission onto dopamine neurons in the ventral tegmental area of the rat. *Alcohol. Clin. Exp. Res.* **32**, 1040–8.

Theile J. W., Morikawa H., Gonzales R. A., Morrisett R. A. (2009) Role of 5-hydroxytryptamine2C receptors in Ca2+-dependent ethanol potentiation of GABA release onto ventral tegmental area dopamine neurons. *J. Pharmacol. Exp. Ther.* **329**, 625–33.

Timmerman W., Westerink B. H. (1997) Brain microdialysis of GABA and glutamate: what does it signify? *Synapse* **27**, 242–61.

Tritsch N. X., Oh W.-J., Gu C., Sabatini B. L. (2014) Midbrain dopamine neurons sustain inhibitory transmission using plasma membrane uptake of GABA, not synthesis. *Elife* **3**, e01936.

Valenta J. P., Job M. O., Mangieri R. A., Schier C. J., Howard E. C., Gonzales R. A. (2013) μ-opioid receptors in the stimulation of mesolimbic dopamine activity by ethanol and morphine in Long-Evans rats: a delayed effect of ethanol. *Psychopharmacology (Berl)*. **228**, 389–400.

van der Zeyden M., Oldenziel W. H., Rea K., Cremers T. I., Westerink B. H. (2008) Microdialysis of GABA and glutamate: analysis, interpretation and comparison with microsensors. *Pharmacol. Biochem. Behav*. **90**, 135–47.

Vetter C. S., Doremus-Fitzwater T. L., Spear L. P. (2007) Time course of elevated ethanol intake in adolescent relative to adult rats under continuous, voluntary-access conditions. *Alcohol. Clin. Exp. Res*. **31**, 1159–68.

Vihavainen T., Relander T. R., Leiviskä R., Airavaara M., Tuominen R. K., Ahtee L., Piepponen T. P. (2008) Chronic nicotine modifies the effects of morphine on extracellular striatal dopamine and ventral tegmental GABA. *J. Neurochem*. **107**, 844–54.

Vinson P. N., Justice J. B. J. (1997) Effect of neostigmine on concentration and extraction fraction of acetylcholine using quantitative microdialysis. *J. Neurosci. Methods* **73**, 61–7.

Volpicelli J. R., Alterman A. I., Hayashida M., O'Brien C. P. (1992) Naltrexone in the treatment of alcohol dependence. *Arch. Gen. Psychiatry* **49**, 876–80.

Walker B. M., Koob G. F. (2007) The γ-aminobutyric acid-B receptor agonist baclofen attenuates responding for ethanol in ethanol-dependent rats. *Alcohol. Clin. Exp. Res.* **31**, 11–8.

Wanat M. J., Sparta D. R., Hopf F. W., Bowers M. S., Melis M., Bonci A. (2009) Strain specific synaptic modifications on ventral tegmental area dopamine neurons after ethanol exposure. *Biol. Psychiatry* **65**, 646–53.

Westerink B. H. C., de Vries J. B. (1989) On the origin of extracellular GABA collected by brain microdialysis and assayed by a simplified on-line method. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **339**, 603–7.

Witt E. D. (2010) Research on alcohol and adolescent brain development: opportunities and future directions. *Alcohol* **44**, 119–24.

Wlodarczyk A. I., Sylantyev S., Herd M. B., Kersanté F., Lambert J. J., Rusakov D. a, Linthorst A. C. E., et al. (2013) GABA-independent GABAA receptor openings maintain tonic currents. *J. Neurosci.* **33**, 3905–14.

Wong C. G. T., Bottiglieri T., Snead O. C. (2003) GABA, gamma-hydroxybutyric acid, and neurological disease. *Ann. Neurol.* **54 Suppl 6**, S3-12.

Wong W. C., Marinelli M. (2016) Adolescent-onset of cocaine use is associated with heightened stress-induced reinstatement of cocaine seeking. *Addict. Biol.* **21**, 634–45.

Xi Z.-X., Ramamoorthy S., Shen H., Lake R., Samuvel D. J., Kalivas P. W. (2003) GABA transmission in the nucleus accumbens is altered after withdrawal from repeated cocaine. *J. Neurosci.* **23**, 3498–505.

Xiao C., Ye J.-H. (2008) Ethanol dually modulates GABAergic synaptic transmission onto dopaminergic neurons in ventral tegmental area: role of μ-opioid receptors. *Neuroscience* **153**, 240–8.

Xiao C., Zhang J., Krnjević K., Ye J. H. (2007) Effects of ethanol on midbrain neurons: role of opioid receptors. *Alcohol. Clin. Exp. Res.* **31**, 1106–13.

Yan Q.-S., Zheng S.-Z., Feng M.-J., Yan S.-E. (2005) Involvement of 5-HT1B receptors within the ventral tegmental area in ethanol-induced increases in mesolimbic dopaminergic transmission. *Brain Res.* **1060**, 126–37.

Yim H. J., Gonzales R. A. (2000) Ethanol-induced increases in dopamine extracellular concentration in rat nucleus accumbens are accounted for by increased release and not uptake inhibition. *Alcohol* **22**, 107–15.

Zalewska-Kaszubska J., Gorska D., Dyr W., Czarnecka E. (2008) Voluntary alcohol consumption and plasma beta-endorphin levels in alcohol-preferring rats chronically treated with naltrexone. *Physiol. Behav.* **93**, 1005–10.

Zhang S., Takeda Y., Hagioka S., Takata K., Aoe H., Nakatsuka H., Yokoyama M., Morita K. (2005) Measurement of GABA and glutamate in vivo levels with high sensitivity and frequency. *Brain Res. Protoc.* **14**, 61–6.

Zhou Y., Danbolt N. C. (2013) GABA and glutamate transporters in brain. *Front. Endocrinol. (Lausanne).* **4**, 165.

Vita

Shannon Laine Zandy received her Doctor of Pharmacy degree from the University of Florida in May 2010. She began her graduate studies in the Division of Pharmacology and Toxicology in the College of Pharmacy at the University of Texas at Austin in June 2010. During her graduate school tenure, she served in leadership positions in graduate student organizations and on the membership committee of the Research Society on Alcoholism. She was the recipient of several awards including Pharmacy Graduate Student Association travel awards, Graduate School Professional Development awards, Graduate School Continuing Fellowship, Johnson & Johnson Endowed Graduate Fellowship and a Pre-doctoral Fellowship in Pharmaceutical Sciences from American Foundation for Pharmaceutical Education. Shannon is continuing her training as an Interdisciplinary Addiction Treatment Postdoctoral Fellow at the Boston Veteran's Administration Medical Center in Boston, Massachusetts.

Permanent email address: szandy@utexas.edu

This dissertation was typed by Shannon Laine Zandy.